# GAS-LIQUID CHROMATOGRAPHIC DETERMINATION OF DRUGS IN PHARMACEUTICALS

by

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# ABSTRACT

Gas-liquid chromatographic methods for the simultaneous determination of eight active ingredients in cough-cold syrups and for the simultaneous determination of three alkaloids in pharmaceutical preparations have been developed. The active ingredients present in cough-cold syrups under study were bromhexine, chlorpheniramine, codeine, dextromethorphan, diphenhydramine, ephedrine/pseudoephedrine, guaiphenesin, and papaverine. And the alkaloids under study were atropine/hyoscyamine, homatropine, and hyoscine.

In both methods, the active ingredients in cough-cold syrups or the alkaloids in eye drops, injections and tablets were first separated from the excipients with chloroform from alkaline medium before injection.

The drugs extracted from the cough-cold syrups were separated by gas-liquid chromatography on a glass column (5 ft. x 2 mm. i.d.) packed with 3% OV-25 supported on Supelcoport (80/100 mesh). The column temperature was maintained at 170 °C for 1 min., then programmed to 265 °C at a rate of 10 °C/min., and maintained at this temperature for 10 min. and 1 min., respectively, for samples with and without papaverine. Clomipramine hydrochloride was used as the internal standard.

The extracted alkaloids were resolved by gas-liquid chromatography on a glass column (5 ft. x 2 mm. i.d.) packed with 3% OV-225 supported on Supelcoport (80/100 mesh). The column temperature was maintained at 220 °C for 1 min., then programmed to 260 °C at a rate of 15 °C/min., and maintained at this temperature for 4 min. and 2 min., respectively, for samples with and without hyoscine. Diphenhydramine hydrochloride was used as the internal standard.

All the chromatographic signals were measured by a flame ionization detector, with an air and hydrogen flow rate of 300 and 30 ml/min., respectively. The nitrogen carrier gas flow rate was set at 30 ml/min. for both methods.

The recoveries for analysis of drugs in the cough-cold syrups ranged from 96.0 to 99.7%, and the relative standard deviations of ten replicate determinations ranged from 0.49 to 4.7%. The method was applied to the determination of active ingredients in nine commercially available cough-cold syrups.

The recoveries for the analysis of alkaloids ranged from 95.9 to 102.1%, and the relative standard deviations of ten replicate determinations ranged from 1.5 to 3.3%. Results are reported for seven commercially available pharmaceutical preparations, including eye drops, injections, and tablets.

An established HPLC method has been modified to enable guaiphenesin in cough-cold syrups to be analysed by isocratic reversed-phase ion-pair high-performance liquid chromatography using an octadecylsilane column. The details are reported in the Appendix.

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 REVIEW OF GAS-LIQUID CHROMATOGRAPHY

In 1941, the concept of gas-liquid chromatography (GLC) was first introduced by Martin and Synge[1a]. In gas chromatography, the sample is injected and then vaporized on the head of a chromatographic column. Elution is brought about by the flow of an inert gaseous mobile phase. Different from most other types of chromatography, the mobile phase does not interact with molecules of the analyte, its only function being to transport those molecules through the packing.

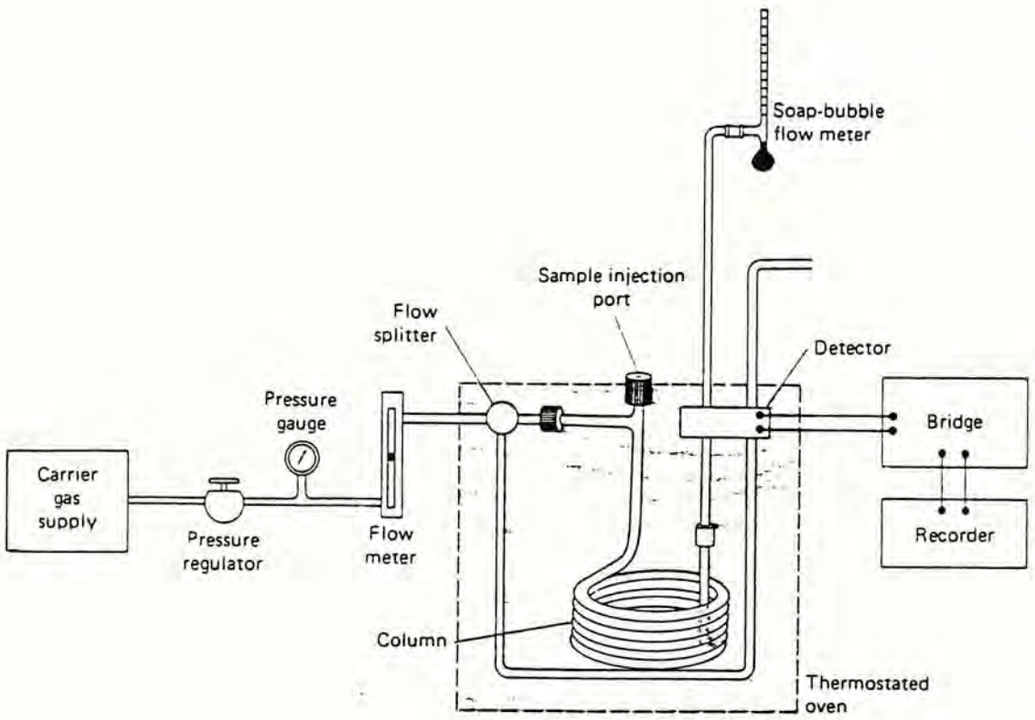
Gas chromatography is a technique for separating volatile compounds by percolating a gas stream over a stationary phase[2]. If the stationary phase is a liquid, we speak of *Gas-liquid Chromatography (GLC)*. The liquid is spread as a thin film over an inert solid and the basis for separation is the partitioning of the sample in and out of this liquid film. The wide temperature range (up to 400 °C) of liquid phases makes GLC the most versatile and selective form of gas chromatography.

The basic components for gas-liquid chromatography are shown in Figure 1.1-1[1b], and a description of each component is as follows:

a. Carrier gas supply[1b]

Carrier gases must be chemically inert, which include argon, helium, hydrogen and nitrogen. The choice of gases is usually dictated by the type of detector used. Flow rates of gases are controlled by a pressure regulator. Inlet pressures usually range from 10 to 50 psi (above room temperature), which lead to flow rates of 25 to 150 ml/min.

**Figure 1.1-1** Schematic diagram of a gas chromatograph.




b. Sample Injection System[1b]

Since column efficiency requires that the sample be of suitable size and be introduced as a "plug" of vapour, a microsyringe is used to inject liquid samples through a rubber or silicone diaphragm or septum into a heated sample port (or injection port) located at the head of the column. The injection port temperature is usually about 50 °C above the boiling point of the least volatile component of the sample. For packed column, the injection volume is varied from a few tenths of microliter to 20 $\mu$l.

c. Columns

Two types of columns are encountered in gas-liquid chromatography, including **packed and capillary.** The former was used in the research project and will be discussed in detail. Unlike open tubular column, the packed column can accommodate larger sample size and are generally more convenient to use.

c.1 *Column dimensions*[1c]

Nowadays, packed columns are usually made by glass or metal (stainless steel, copper, or aluminum) tubes with lengths of 2 to 3 m and inside diameters of 2 to 4 mm. The tubes are usually in the form of coils having diameter of around 15 cm.

c.2 *Types of solid supports*[1c]

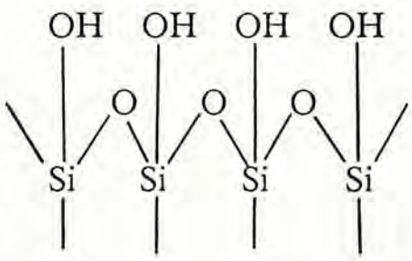
The solid support used to hold the liquid stationary phase in place so that as large a surface area as possible is exposed to the mobile phase. The most widely used supports for gas chromatography were prepared from naturally occurring diatomaceous earth. The solid support may be prepared by crushing, blending, and briquetting the diatomaceous earth as it comes from the ground followed by heating at over 900 °C. The resulting bricks are then ground and separated by scanning into particles having uniform diameters. An example is Chromosorb P. Alternately, the solid support (e.g. Chromosorb W) may be prepared by mixing the diatomaceous earth with a sodium carbonate flux before heating at about 900 °C. The product is more rugged than the former, however, its specific surface area is only about 1 $m^2/g$ compared with 4 $m^2/g$ for the former.

c.3 *Particle size of supports*

Although the column efficiency increases rapidly with decreasing particle diameter of the packing, the pressure difference required to maintain a given flow-rate of carrier gas varies inversely as the square of the particle diameter[1c]. This relationship has placed lower limits on the size of particles employed in gas chromatography since it is inconvenient to use pressure differences that are greater than about 30 psi. As a result, the usual support particles are 60 to 80 mesh (250 to 170 $\mu$m) or 80 to 100 mesh (170 to 149 $\mu$m).

c.4 *Adsorption on solid supports*[1d]

Physical adsorption on support surfaces of polar or polarizable analyte species results in distorted peaks, which are broadened and often exhibit a tail. Adsorption is the consequence of the formation of silanol groups on the surface of silicate by the reaction with moisture. A fully hydrolysed silicate surface has the structure




The SiOH groups have a strong affinity for polar organic molecules and tend to retain them by adsorption.

Support materials can be deactivated by silanization with dimethylchlorosilane (DMCS) or hexamethyldisilazane (HMDS).

c.5 *Stationary phase*

The ideal stationary phase in a gas chromatographic column should have the properties as follows:**(1) low volatility; (2) thermal stability; (3) chemical inertness; and (4) solvent characteristics**[1d]. Even though the choice of stationary phase could base upon polarity parameters of the stationary phase relative to those of the sample constituents, the optimal separation conditions can only be realized by trial-and-error experiments.

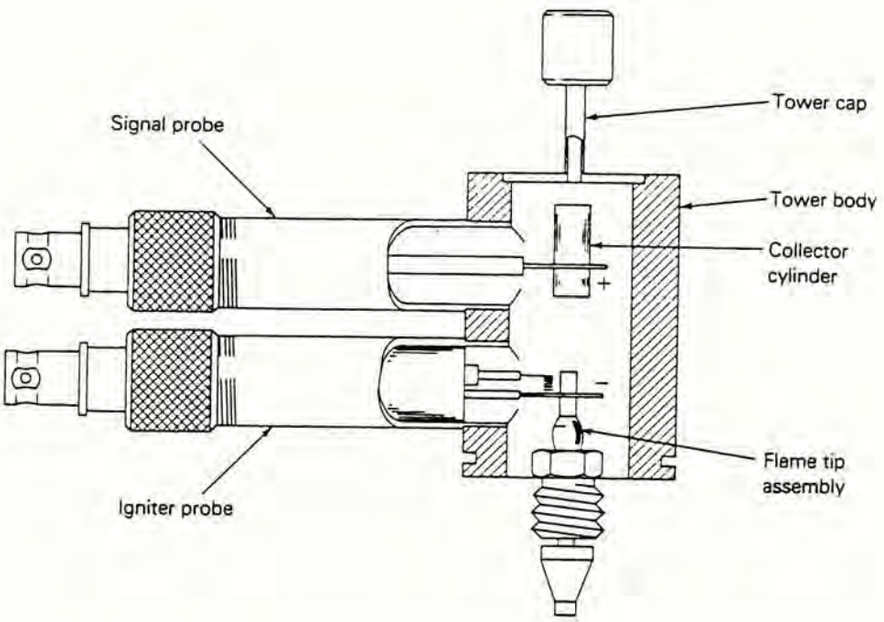
McReynolds constants[1e] are symbolized by X', Y', Z', U' and S'. Each is related to one type of interaction between a solute and the stationary phase, i.e. X'=benzene, Y'=1-butanol, Z'=methyl-n-propyl ketone, U'=nitropropane, and S'=pyridine. The magnitude of each of them provides a measure of the strength of this type of interaction for a group of compounds represented by the standard. For example, aromatics and olefins have the same polarity characteristics as benzene. As a result, compounds containing those functional groups will be most strongly retained by a stationary phase having a large value of X'. McReynolds constants are widely used to characterize the multitude of stationary phases that are now available. One of the important uses of McReynolds numbers is for recognizing similar liquid phases in order to avoid duplication of effort in testing the column efficiency for a given separation.

d Detectors[1f]

Numerous detectors have been investigated and used during the development of gas chromatography. However, only four have found widespread use, including thermal conductivity, flame ionization, thermionic, and electron capture. Details of a flame ionization detector is described here because it has been employed in the present research project.

The flame ionization detector (FID) was first introduced in 1958. Its high sensitivity, uniform response to hydrocarbons, and a broad linear range have made the FID become one of the most popular measuring device used in gas chromatography. Use of the FID is based on the measurement of variations in the ionization current in a hydrogen-air flame due to the presence of eluted substances. With a burner such as that shown in Figure 1.1-2, the charged species are attracted to and captured by a collector; an ion current results, which can be amplified and recorded.

**Figure 1.1-2** A typical flame ionization detector.




Although many questions about the flame chemistry in the FID remain unsolved, it was suggested that the primary source of ions in the flame was produced through a chemical ionization caused by the reaction of an oxygen atom with a radical containing a single carbon[3,4]. The most commonly proposed reaction is

$$CH + O \longrightarrow CHO^{+} + e^{-}$$

It is observed that the number of ions produced is roughly proportional to the number of *reduced carbon* atoms (or *effective carbon number, ECN*) in the plasma. Functional groups, such as carbonyl, alcohol, halogen, and amine, yield fewer ions or none at all. Besides, the detector is insensitive towards noncombustible gases such as $H_2O$, $CO_2$, $SO_2$, and $NO_x$. This behaviour makes the flame ionization detector a most useful general detector for the analysis of most organic samples.

Recently, Jorgensen et al[4] has attempted to develop a computer program that would query the user for structural information about each peak in a chromatogram, calculate the ECN from information in a data base, and use the area and concentration of the internal standard to calculate the concentration of the component.

The performance of the flame ionization detector depends on the proper choice of gas flow rates[5a]. In general, good sensitivity and stability are obtained with a carrier gas flow of 30 ml/min, hydrogen flow of 30 ml/min and air flow at 300 ml/min. Figure 1.1-3[5b] shows the relation between FID sensitivity and hydrogen flow rate, and Figure 1.1-4[5c] shows the relation between FID sensitivity and air flow rate. In addition, the flame ionization detector exhibits a high sensitivity (about $10^{-13}$ g/ml), large linear range (about $10^7$), and low noise. A demerit of the flame ionization detector is that it is destructive of the sample.

**Figure 1.1-3** Relationship between FID sensitivity and hydrogen flow rate.

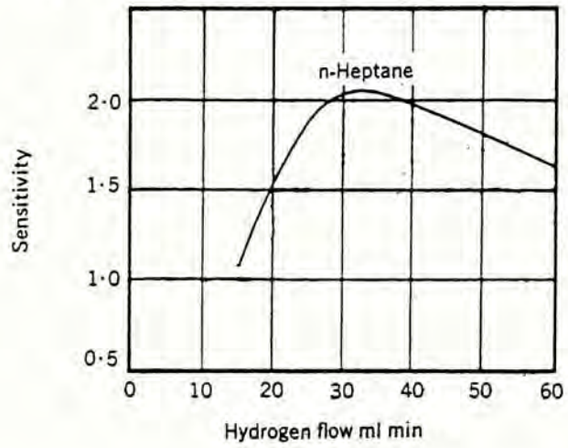
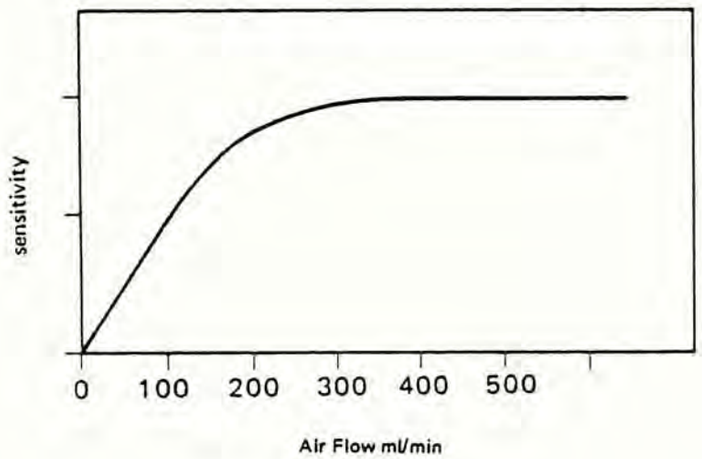



**Figure 1.1-4** Relationship between FID sensitivity and air flow rate.




## 1.2 APPLICATION OF GAS-LIQUID CHROMATOGRAPHY IN DRUG ANALYSIS[6]

All drug analyses virtually require both identification and quantitation. In this regard, gas chromatography offers major advantages. The high sensitivity of gas chromatography is an important factor, especially in drug metabolism or toxicity studies, where only trace quantities of drug are available. High-resolution gas chromatographic columns allow excellent selectivity in the presence of structurally similar analogs or degradates. Besides, the ability of adjusting parameters such as carrier gas flow and column temperature enables the analyst to optimize selectivity.

Many drugs are non-volatile compounds. Besides, they contain relatively polar substituent groups which contribute to interaction with the solid support. In order to overcome these problems, derivatization technique was commonly used in the past two decades to prepare thermally stable and volatile compounds for gas chromatographic analysis. Cimbura and Kofoed[7] had given a review of some GLC-FID derivatization techniques, including acetylation, methylation, and chemical reduction, which were found useful in forensic toxicology. Cruickshank and Sheehan[8] demonstrated the use of gas chromatography in analysis of 21 amino acids as their N-trifluoroacetylamino acid methyl esters. Parker et al[9] described the chromatographic behaviours of 41 alkaloids on a column of 5% SE-30 at five temperatures. Besides, Kazyak and Knoblock[10] illustrated the application of gas chromatography to analytical toxicology, and the chromatographic behaviours of 59 drugs on 1% SE-30 were reported.

Nowadays, the advances in the development of new types of stationary phases allow the analysts to perform direct gas-liquid chromatographic analysis of drugs without preliminary or on-column derivatization. For example, Gibbs et al[11] and Rao et al[12] reported the use of GLC for quantitative determination of the active ingredients in pharmaceutical preparations as their free basic amine.

## 1.3 AIMS OF THE PRESENT WORK

The aims of the present work were to develop simple and efficient gas-liquid chromatographic methods for the simultaneous determination of active ingredients in cough-cold syrups and for the simultaneous determination of alkaloids in pharmaceutical preparations.

The drugs under study were bromhexine, chlorpheniramine, codeine,

dextromethorphan, diphenhydramine, ephedrine, guaiphenesin, papaverine, and pseudoephedrine, which are commonly found in commercially available cough-cold syrups. The drugs were extracted with chloroform from an alkaline medium, and separated on a glass column packed with 3% OV-25. The details of the proposed method will be discussed in Chapter 2.

The alkaloids under study were atropine, homatropine, and hyoscine, and hyoscyamine, that are usually found in eye drops, injections, and tablets. Those alkaloids were separated from the excipients with chloroform, and resolved by a glass column packed with 3% OV-225. The detailed descriptions will be shown in Chapter 3.

An established HPLC method for quantitative determination of the active ingredients in cough-cold syrups had been modified for the purpose of checking the contents of guaiphenesin in cough-cold syrups. The results are reported in the Appendix.

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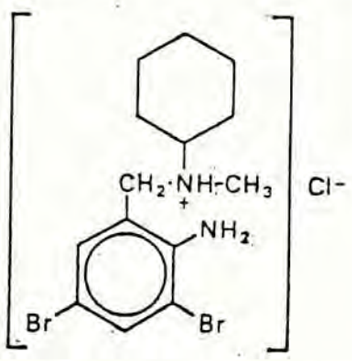
# CHAPTER 2

# GAS-LIQUID CHROMATOGRAPHIC DETERMINATION OF ACTIVE INGREDIENTS IN COUGH-COLD SYRUP FORMULATIONS

## 2.1 INTRODUCTION

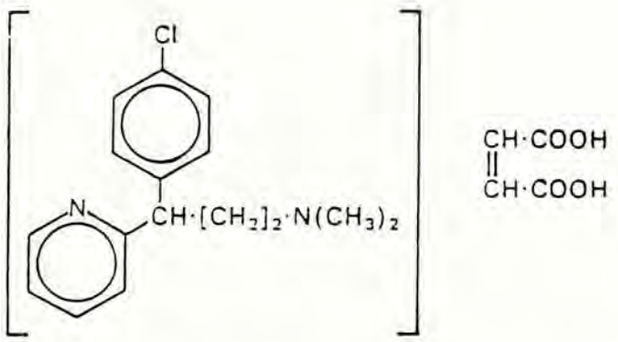
Bromhexine, chlorpheniramine, codeine, dextromethorphan, diphenhydramine, ephedrine, guaiphenesin, papaverine and pseudoephedrine are active ingredients commonly found in cough-cold syrups. Each of them possesses its particular functions[1,2]:

i. *Bromhexine*



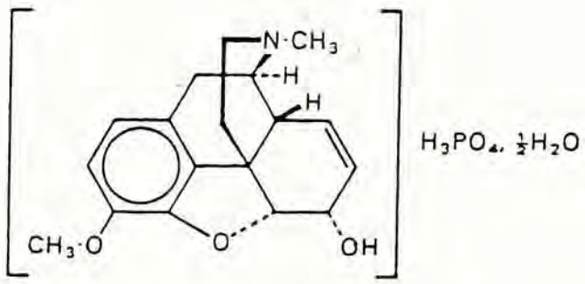

Bromhexine is a mucolytic agent which changes the structure of bronchial secretions by rarefication and fragmentation of the mucopolysaccharide fibres, leading to a reduction in the viscosity of the sputum.

ii. *Chlorpheniramine*



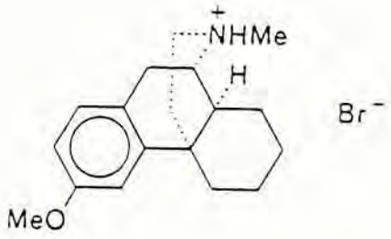

Chlorpheniramine has the actions, uses and undesirable effects of the antihistamine drugs and is effective in smaller doses but it has no marked anti-emetic effect.

iii. *Codeine*



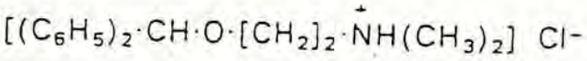

Codeine is an analgesic with uses similar to those of morphine but it is much less potent as an analgesic and has only mild sedative effects. It possesses very useful anti-tussive effect, and hence, it is included in most anti-cough mixtures.

iv. *Dextromethorphan*



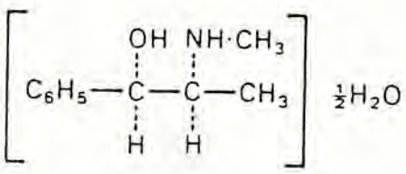

Dextromethorphan has a depressant action on the cough centre similar to that of codeine phosphate, but it has no analgesic or expectorant effect. Its use does not lead to addiction.

v. *Diphenhydramine*



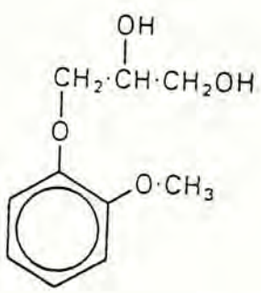
Diphenhydramine has the actions, uses, and undesirable effects of antihistamine drugs. It is one of the less active but more sedating of the group and is sometimes used for its sedative properties.

vi. *Ephedrine*



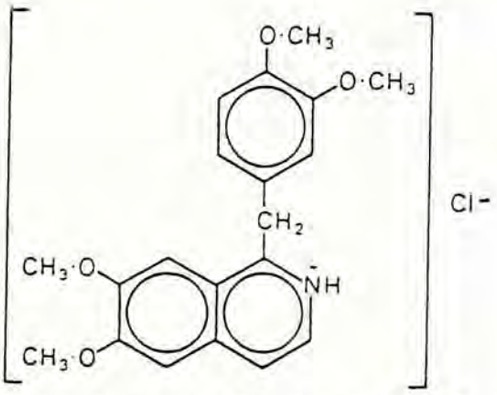
Ephedrine is a sympathomimetic amine with direct and indirect effects on adrenoreceptors.

vii. *Guaiphenesin*



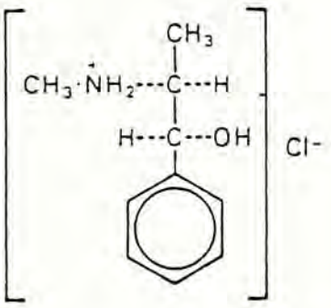
Guaiphenesin is reported to reduce the viscosity of tenacious sputum and is used as an expectorant in cough mixtures and tablets.

viii. *Papaverine*




Papaverine has little hypnotic or analgesic action. It is used occasionally to produce relaxation of involuntary muscles.

ix. *Pseudoephedrine*




Pseudoephedrine is a sympathomimetic amine with action and undesirable effects resembling those of ephedrine. It is used as a bronchodilator and peripheral vasoconstrictor in preparations for the relief of nasal and bronchial congestion, particularly in bronchial asthma.

High-performance liquid chromatography (HPLC)[3-6] are commonly used for the determination of the active ingredients in cough-cold mixtures. Besides, derivative spectrophotometric methods[7-9] and gas-liquid chromatographic (GLC) methods[4,5,10] have also been developed for the analysis of these drugs, however, most of them could only quantitatively determine two or three components simultaneously. In addition, the methods involving the measurement of UV absorption were easily interfered by the excipients present in cough syrups, such as dyes and flavours.

The present work is to develop a simple and efficient gas-liquid chromatographic method for simultaneous determination of the eight* active ingredients mentioned above, individually or together, in various cough-cold syrup formulations after extraction of the active ingredients with chloroform. The optimum conditions for carrying out the extraction and for the measurements were determined. Nine commercially available cough-cold syrups were analysed for the contents of the active ingredients, and the results were compared with those obtained using high-performance liquid chromatography[3]. In performing the counter-check method, it was found that this method was also suitable for the determination of guaiphenesin in cough-cold syrup formulations after a minor modification, and the results are shown in the Appendix.

*Ephedrine and pseudoephedrine being stereoisomers have similar properties and are not found together in cough-cold syrups.

## 2.2 EXPERIMENTAL

### 2.2.1 Instrumentation

#### 2.2.1.1 *Apparatus*

A Varian Model 3700 Gas Chromatograph equipped with a flame ionization detector, and attached to a Hitachi 833A Data Processor was used.

2.2.1.2 *Gas chromatographic conditions*

A glass column (5ft. x 2 mm i.d.) was packed with 3% OV-25 on Supelcoport (80/100 mesh). The carrier gas was nitrogen with a flow rate of 30 ml/min. The flow rate of hydrogen and air were 30 and 300 ml/min., respectively. The injection port and detector temperatures were both kept at 270 °C. The initial column temperature was kept at 170 °C for the first minute, then programmed to a final temperature of 265 °C at a heating rate of 10 °C/min., and then maintained at this temperature for 10 min. and 1 min., respectively, for samples with and without papaverine. The attenuation was 1 in the gas chromatograph and varied in the data processor (8-10, depending on the injection concentration). The chart speed of the data processor was 2.5 mm/min. When not in use, the column was kept at 50 °C with a nitrogen flow rate of 30 ml/min. When broadening of the peaks was observed, which was an indication of decomposition of the packing material, the first few inches of the packing material near the injection port was replaced, and the column could be used for about six months.

2.2.2 The Counter-check HPLC Method

An established HPLC method[3] was found to be suitable for checking the contents of all active ingredients under study except guaiphenesin in cough-cold syrups. With a minor modification of the HPLC method, it can be used to check the content of guaiphenesin in cough-cold syrups and the details of the modification are shown in the Appendix.

2.2.2.1 *Instrumentation*

The liquid chromatograph consisted of a controller (Beckman, model 421A), a solvent pump (Beckman, model 110B), an injection system (Altex, 210 valve), an

analytical column Beckman 5 $\mu$ Ultrasphere-ODS 250 x 4.6 mm, i.d., a detector (Beckman, model 163 variable wavelength) and an integrator (Beckman, model 427). The column was protected by a guard column packed with the same packing material.

Instrumental settings

| | Original | For guaiphenesin |
|---|---|---|
| Flow rate | 1.3 ml/min | 1.0 ml/min. |
| Wavelength | 254 nm | 274 nm |
| Chart speed | 0.5 cm/min. | 0.5 cm/min. |
| Attenuation | 16 | 16 |

2.2.2.2 *Mobile phase*

Composition of the mobile phase:

| | Original | For guaiphenesin |
|---|---|---|
| Methanol | 715 ml | 635 ml |
| Water | 234 ml | 315 ml |
| Tetrahydrofuran | 50 ml | 50 ml |
| Phosphoric acid (85%) | 1 ml | 1 ml |
| Sodium dioctyl-sulphosuccinate | 5.8 g | 5.8 g |
| pH (adjusted by ammonia) | 4.6 | 4.6 |

2.2.3 Reagents

All drugs were of Pharmacopoeial or equivalent purity, and were used without further purification. All other reagents were of analytical grade.

2.2.3.1 *Aqueous internal standard solution*

Internal standard solution of clomipramine hydrochloride (1.502 mg/ml) was prepared by dissolving exactly 75.1 mg of the compound in 50 ml of distilled water in a volumetric flask.

2.2.3.2 *Aqueous stock solutions of the drugs*

Stock solution of bromhexine hydrochloride (2.50 mg/ml) was prepared by weighing exactly 125.00 mg of the drug in 50 ml of sulphuric acid (0.1M) in a volumetric flask.

Similarly, stock solutions of chlorpheniramine maleate (1.02 mg/ml), codeine phosphate (5.00 mg/ml), dextromethorphan hydrobromide (3.12 mg/ml), diphenhydramine hydrochloride (5.00 mg/ml), ephedrine hydrochloride (5.00 mg/ml), guaiphenesin (15.63 mg/ml), papaverine hydrochloride (1.25 mg/ml) and pseudoephedrine hydrochloride (6.24 mg/ml) were prepared by dissolving appropriate amounts of the corresponding drugs in 50 ml of sulphuric acid (0.1M) in volumetric flasks.

2.2.4 Aqueous Standard Solutions

Various mixtures of drugs were prepared by mixing appropriate amounts of stock solutions of these drugs to match the active ingredients and their respective label values for each syrup. For example, for sample No. 4 in Table 2.3.6-2 (page 69) containing

codeine phosphate (9.0 mg/5ml) and ephedrine hydrochloride (7.2 mg/5ml), a mixture was prepared by mixing accurately 10 ml each of the codeine phosphate and ephedrine hydrochloride stock solutions and diluting to 25 ml in a calibrated flask, the resulting concentrations for both drugs in this mixture being 10.0 mg/5ml. Other mixtures with different compositions were prepared in the same way.

### 2.2.5 Sample Pre-treatment

No sample pre-treatment was necessary except for samples No. 7-9 (page 69-70) containing high concentrations of the drugs and they were 5-fold diluted with 0.1M sulphuric acid.

### 2.2.6 Extraction Method

10 ml of the cough-cold syrup or aqueous standard solution was pipetted into a 100-ml separating funnel with addition of equal amount of 25 % V/V ammonia solution (Analar-grade, Merck). The drugs were extracted three times with 15-ml aliquots of chloroform. The organic layers were transferred to another separating funnel and washed with 5-6 ml of distilled water. The chloroform layer was collected into a 50-ml V-flask via a funnel containing about 5 g of anhydrous sodium sulphate supported by a filter paper (No. 5A). The sodium sulphate on the filter was washed with small volumes of chloroform, and the washings were added to the filtrate, which was then diluted to mark with chloroform.

### 2.2.7 Preparation of Working Internal Standard Solution

An aliquot (10 ml) of the aqueous internal standard solution was treated as for the cough-cold syrups.

2.2.8 Simultaneous Determination of Active Ingredients in Cough-cold Syrups

An aliquot (5 ml) of the sample solution and several aliquots (2-10 ml) of the standard solution after the extraction procedure described above were evaporated to dryness under a stream of nitrogen in a water bath at room temperature. Each residue was dissolved in exactly 1 ml of the working internal standard solution, 3 $\mu$l of which were injected into the chromatograph with a 10-$\mu$l Hamilton syringe under the chromatographic conditions described above. The injections were performed in duplicate.

The calibration graph was obtained by plotting the peak area ratios (drug to internal standard) against the concentrations of the drug in the injected standard solutions.

The concentration of each drug in the injected sample solution was deduced from the respective calibration graph, and the amount of each drug in the original sample was then calculated.

## 2.3 RESULTS AND DISCUSSION

### 2.3.1 Choice of Extraction Medium

Because of the presence of dye(s), preservative(s), flavour(s) and a sweetening agent in most of the cough-cold syrups, the quantitative determination of the active ingredients in cough-cold mixtures become quite difficult. In order to analyse the active ingredients in cough-cold mixtures by gas chromatography, an efficient extraction method should be developed for separating the active ingredients from the other excipients.

The drugs under study are reported to be extracted by organic solvents from alkaline media[11,12]. Thus, the aqueous solutions of the drugs were first made alkaline with 25% V/V ammonia solution before extraction. Several common organic solvents, including chloroform, dichloromethane, diethylether and hexane, were selected for the evaluation of their suitability as the extraction medium.

An aqueous standard solution, containing chlorpheniramine maleate (0.4700 mg/ml), codeine phosphate (2.276 mg/ml), dextromethorphan hydrobromide (1.320 mg/ml), ephedrine hydrochloride (1.542 mg/ml), and guaiphenesin (3.042 mg/ml), was prepared. Aliquots (2 ml) of the aqueous standard solution were made alkaline with ammonia solution and extracted once with exactly 20 ml of each solvent, and 10 ml of the organic layer was pipetted out and filtered after being dried with anhydrous sodium sulphate. The filtrate was evaporated to dryness under a stream of nitrogen, the residue was dissolved in exactly 1 ml of the working internal standard solution, and then 3.0 $\mu$l of the final solution was injected onto the chromatograph. The peak area ratios (drug to internal standard) of each compound in various organic solvents are shown in Table 2.3.1-1.

Since the peak area ratios so obtained were directly proportional to the amounts of drugs extracted from the aqueous to the organic phase, these ratios were used to compare the extraction capability of each solvent. The result shown in Table 2.3.1-1 indicated that chloroform should be the best extraction medium while hexane was the poorest one. It is worthwhile to note that the extraction capability of chloroform was enhanced by the addition of 10% (V/V) of methanol. Unfortunately, this mixed solvent also extracted the dyes in real samples. Therefore, methanol was not included in the subsequent experimental procedure, and chloroform was finally chosen to be the extraction medium.

**Table 2.3.1-1** Peak area ratios (individual drug to internal standard) after extraction with various solvents*

| Extraction medium | Peak area ratios of the drugs in the mixture+ | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Chloroform | 3.405 | 4.332 | 0.4452 | 2.777 | 3.823 |
| Dichloromethane | 2.787 | 3.807 | 0.3307 | 2.421 | 3.791 |
| Diethylether | 2.694 | 1.548 | 0.2285 | 2.229 | 2.870 |
| Hexane | 1.055 | n.d.** | 0.3543 | 2.277 | 0.7384 |
| 10% MeOH in $CHCl_3$ | 3.436 | 4.724 | 0.4308 | 2.850 | 4.234 |

*Chromatographic conditions as described in the Experimental section (section 2.2);

+The drugs in the mixture, with concentration in the organic solvent in parentheses:

(1) Ephedrine hydrochloride (1.542 mg/ml);

(2) Guaiphenesin (3.042 mg/ml);

(3) Chlorpheniramine maleate (0.4700 mg/ml);

(4) Dextromethorphan hydrobromide (1.320 mg/ml);

(5) Codeine phosphate (2.276 mg/ml).

**No guaiphenesin signal was detected.

After choosing the extraction medium, the number of portions of chloroform used for extraction became the key point to determine the efficiency and simplicity of the proposed extraction method. Thus, the percentages extraction of each drug under study obtained by extraction with 3 x 15 ml and 5 x 15 ml of chloroform were deduced by UV spectrophotometric method with the following procedure:

A standard solution of each drug was prepared by dissolving appropriate amount of the compound in 0.1M sulphuric acid, 10 ml of which was pipetted into each of two 100-ml separating funnels, namely I and II, and both were made alkaline with ammonia. The drug in separating funnels I and II were extracted by three portions and five portions of 15 ml of chloroform, respectively. The organic layers in both separating funnels were dried with anhydrous sodium sulphate and filtered, and the filtrate and washings from I and II were made up to 50-ml and 100-ml, respectively, in calibrated flasks. From each of the calibrated flasks, portions of the solutions (5 ml from the 50-ml V-flask and 10 ml from the 100-ml V-flask) were separately pipetted out and evaporated to dryness under a stream of nitrogen in a water bath. The residues were dissolved separately in 10 ml of 0.1M sulphuric acid, and their UV absorbances at maximum absorbance wavelengths were measured. The amount of drug in each solution was deduced from the calibration graph. The percentage extraction of individual drug was then calculated. The results are shown in Table 2.3.1-2.

By comparing the percentage extraction of individual drug obtained by 3 x 15 ml and 5 x 15 ml extraction, no significant difference in the extraction efficiency was detected. As a result, three portions of 15 ml of chloroform were used in the proposed extraction method in order to save time and reagent.

**Table 2.3.1-2** Percentage extraction of individual drug obtained by 3 x 15 ml and 5 x 15 ml extraction.

| Compound | Concentration in aqueous solution (mg/ml) | Percentage extraction (%) | |
|---|---|---|---|
| | | By 3 x 15 ml extraction | By 5 x 15 ml extraction |
| Bromhexine HCl | 0.5520 | 99.2 | 100.2 |
| Chlorpheniramine maleate | 1.028 | 97.4 | 98.1 |
| Codeine phosphate | 1.152 | 95.3 | 96.3 |
| Dextromethorphan HBr | 1.028 | 100.1 | 98.6 |
| Diphenhydramine HCl | 2.008 | 97.1 | 99.5 |
| Ephedrine HCl | 3.004 | 95.1 | 96.4 |
| Guaiphenesin | 1.352 | 90.3 | 90.5 |
| Papaverine HCl | 0.7560 | 99.3 | 100.1 |
| Pseudoephedrine HCl | 2.992 | 99.0 | 99.1 |

2.3.2 Choice of Stationary Phase

As it was intended to resolve a large number of drugs using the proposed method, the proper choice of stationary phase was also important. Since there was no perfect guidelines for choosing the best stationary phase, the optimal separation conditions could only be realized by trial-and-error experiments. A number of stationary phases on Supelcoport (80/100 mesh), including 3% OV-1, 3% OV-17, 3% OV-25 and 3% OV-225, were assessed. The composition, McReynolds constants, and temperature limits of these stationary phases are shown in Table 2.3.2-1. All of them can tolerate high column temperatures (i.e. maximum temperature limits are high), thus, they are suitable for separation of non-volatile drugs of interest. The large difference in McReynolds constants of the various stationary phases indicated that each of them should have different separation power for the drugs under study.

During the study, the flow rate of nitrogen was set at 30 ml/min., which was commonly used for the determination of drugs. The optimum temperature programming conditions for separating the drugs under study were found for each column. The retention time and peak shapes of the drugs under the optimised conditions for each column are listed in Table 2.3.2-2. The typical chromatograms of the drugs obtained by the various columns are shown in Figure 2.3.2-1 to Figure 2.3.2-4. It is noted that only ephedrine but not pseudoephedrine is shown as these two compounds are stereoisomers and have the same peak shape and retention time. They have similar properties and will never co-exist in cough-cold syrups.

Both stationary phases of 3% OV-1 and 3% OV-225 were found to be not suitable for the separation of all eight ingredients. The former gave an overlapping peak of bromhexine HCl and codeine phosphate, while, the latter could not completely resolve the peaks of chlorpheniramine maleate and diphenhydramine HCl. Although

the stationary phase of 3% OV-17 could separate all of the drugs under study, the overall running time was rather long and the resolution was not good enough. In terms of good peak shapes, resolution, and overall running time, the column packed with 3% OV-25 was found to be the most satisfactory one.

**Table 2.3.2-1** The specifications of various stationary phases*.

| Phase | Composition | Temperature limit (°C) | McReynolds constants** | | | | |
|---|---|---|---|---|---|---|---|
| | | Min./Max. | X' | Y' | Z' | U' | S' |
| OV-1 | Dimethyl (gum) | 100/350 | 16 | 55 | 44 | 65 | 42 |
| OV-17 | Phenyl methyl, 50% phenyl | 0/375 | 119 | 158 | 162 | 243 | 202 |
| OV-25 | Phenyl methyl diphenyl, 75% phenyl | 0/350 | 178 | 204 | 208 | 305 | 280 |
| OV-225 | Cyanopropylmethyl -phenyl methyl | 0/265 | 228 | 369 | 338 | 492 | 386 |

*Reference : SUPELCO, International Catalog 25, 1987, pp.88.

**Test solutes :
X' = benzene;
Y' = 1-butanol;
Z' = methyl-n-propyl ketone;
U' = nitromethane; and
S' = pyridine.

**Table 2.3.2-2** The retention times and peak shapes of the drugs under study in various types of column under the conditions specified in Table 2.3.2-2a.

| | Retention time*, min. | | | |
|---|---|---|---|---|
| | Glass column packed with | | | |
| Compounds | 3% OV-1 | 3% OV-17 | 3% OV-25 | 3% OV-225 |
| Ephedrine HCl** Pseudoephedrine HCl | 1.06 (s) | 1.18 (s) | 1.04 (s) | 2.80 (s) |
| Guaiphenesin | 2.87 (s) | 4.59 (s) | 3.67 (st) | 7.30 (s) |
| Diphenhydramine HCl | 4.94 (s) | 6.12 (st) | 4.88 (s) | 9.46 (so) |
| Chlorpheniramine maleate | 6.42 (s) | 7.95 (st) | 6.22 (s) | 9.70 (so) |
| Dextromethorphan HBr | 7.79 (s) | 9.51 (st) | 7.49 (s) | 11.12 (s) |
| Bromhexine HCl | 10.31 (o) | 11.99 (s) | 9.37 (s) | 15.52 (s) |
| Codeine phosphate | 10.31 (o) | 13.27 (s) | 10.84 (s) | 20.01 (s) |
| Papaverine HCl | 14.73 (s) | 22.31 (s) | 19.14 (s) | 30.59 (b) |

* Description of the peak shape was enclosed in parentheses:
b, board; o, overlapped; s, sharp; so, sharp but overlapped; and st, sharp with tailing.

** Ephedrine HCl and pseudoephedrine HCl had the same retention time and peak shape in each type of column.

**Table 2.3.2-2a** Optimised chromatographic conditions for columns in Table 2.3.2-2. The columns (5 ft. x 2 mm i.d.) were packed respectively with the following stationary phases on Supelcoport (80/100 mesh).

| | Stationary phase | | | |
|---|---|---|---|---|
| | 3% OV-1 | 3% OV-17 | 3% OV-25 | 3% OV-225 |
| Initial temperature (°C) | 150 (1 min.) | 160 (1 min.) | 170 (1 min.) | 180 (1 min.) |
| Heating rate (°C/min.) | 8 | 8 | 10 | 6 |
| Final temperature (°C) | 260 (2 min.) | 260 (10 min.) | 265 (10 min.) | 258 (18 min.) |

**Figure 2.3.2-1** Gas chromatogram of the drugs of interest obtained by a column of **3% OV-1** under the optimised conditions. A, solvent; B, ephedrine hydrochloride (2.98 mg/ml); C, guaiphenesin (5.95 mg/ml); D, diphenhydramine hydrochloride (2.50 mg/ml); E, chlorpheniramine maleate (3.03 mg/ml); F, dextromethorphan hydrobromide (1.93 mg/ml); G, bromhexine hydrochloride (1.96 mg/ml) and codeine phosphate (1.93 mg/ml); H, papaverine hydrochloride (2.99 mg/ml).

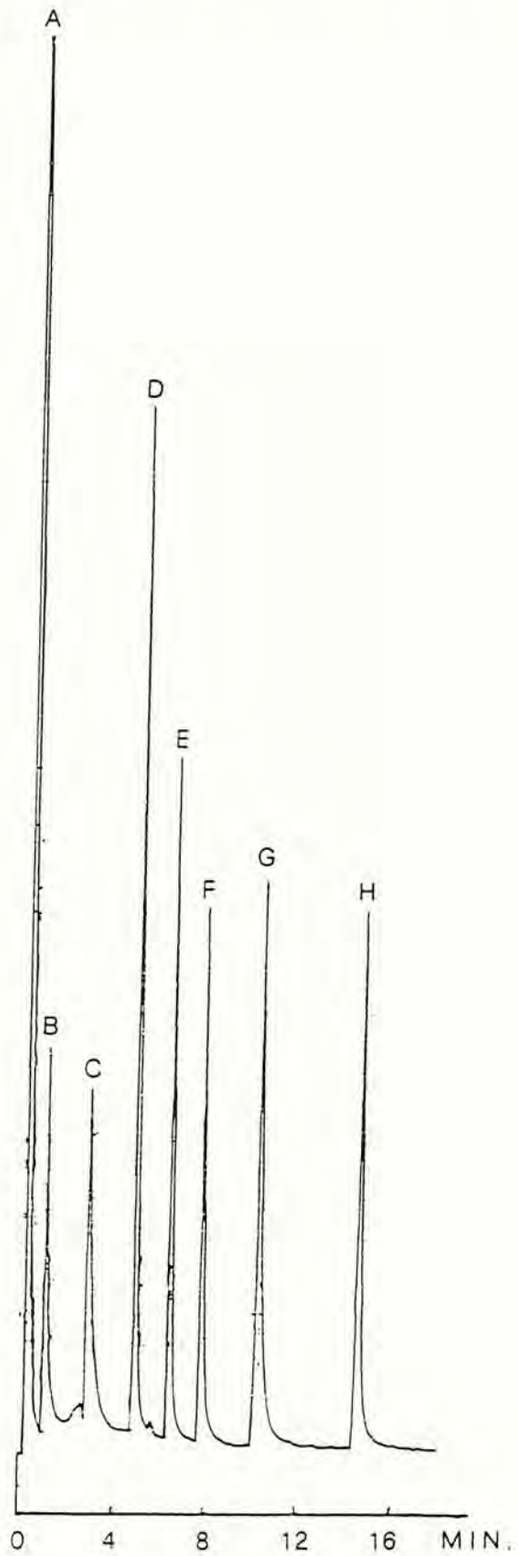


**Figure 2.3.2-2** Gas chromatogram of the drugs of interest obtained by a column of 3% **OV-17** under the optimised conditions. A, solvent; B, ephedrine hydrochloride (2.98 mg/ml); C, guaiphenesin (5.95 mg/ml); D, diphenhydramine hydrochloride (2.50 mg/ml); E, chlorpheniramine maleate (3.03 mg/ml); F, dextromethorphan hydrobromide (1.93 mg/ml); G, bromhexine hydrochloride (1.96 mg/ml), H, codeine phosphate (1.93 mg/ml); and I, papaverine hydrochloride (2.99 mg/ml).

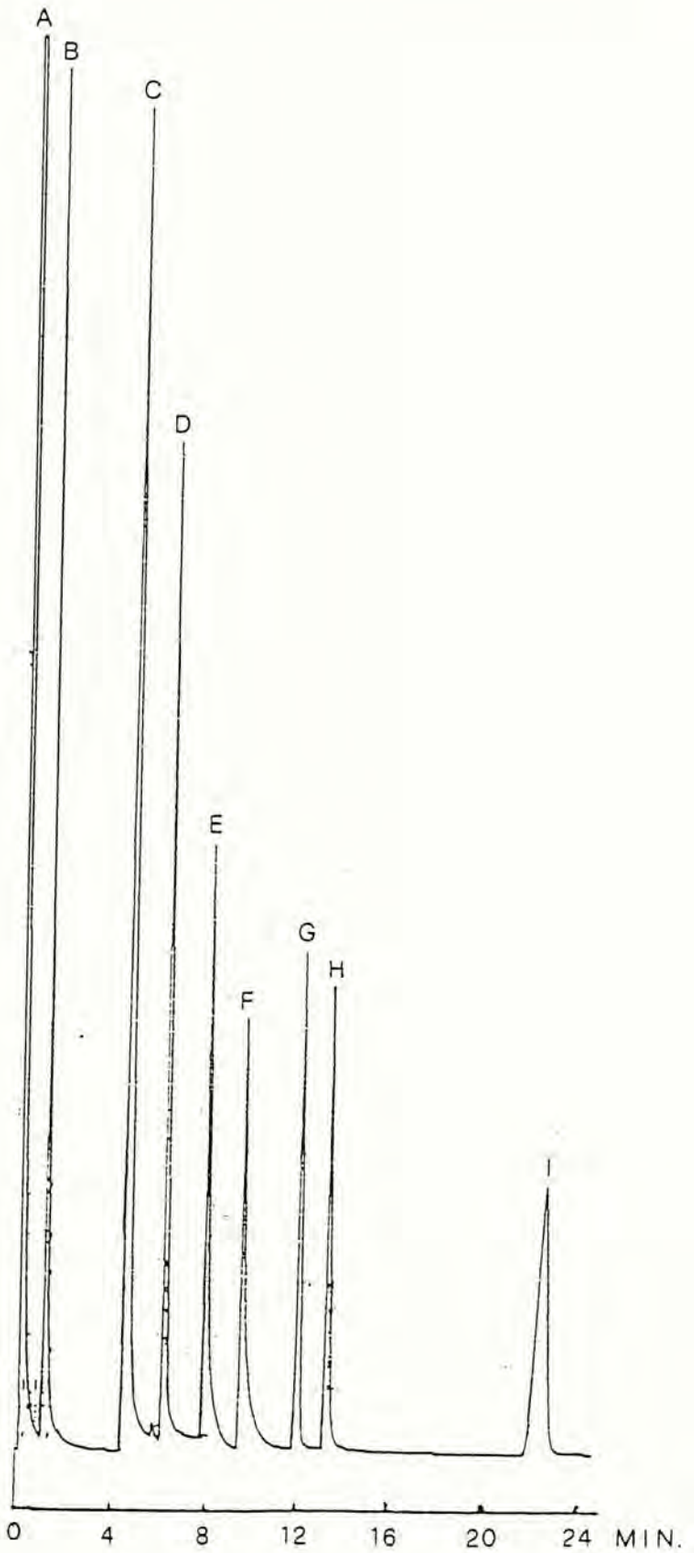


**Figure 2.3.2-3** Gas chromatogram of the drugs of interest obtained by a column of 3% **OV-25** under the optimised conditions. A, solvent; B, ephedrine hydrochloride (0.87 mg/ml); C, guaiphenesin (1.54 mg/ml); D, diphenhydramine hydrochloride (1.09 mg/ml); E, chlorpheniramine maleate (0.90 mg/ml); F, dextromethorphan hydrobromide (1.00 mg/ml); G, bromhexine hydrochloride (0.77 mg/ml); H, clomipramine hydrochloride (0.30 mg/ml); I, codeine phosphate (0.91 mg/ml); and J, papaverine hydrochloride (0.47 mg/ml).

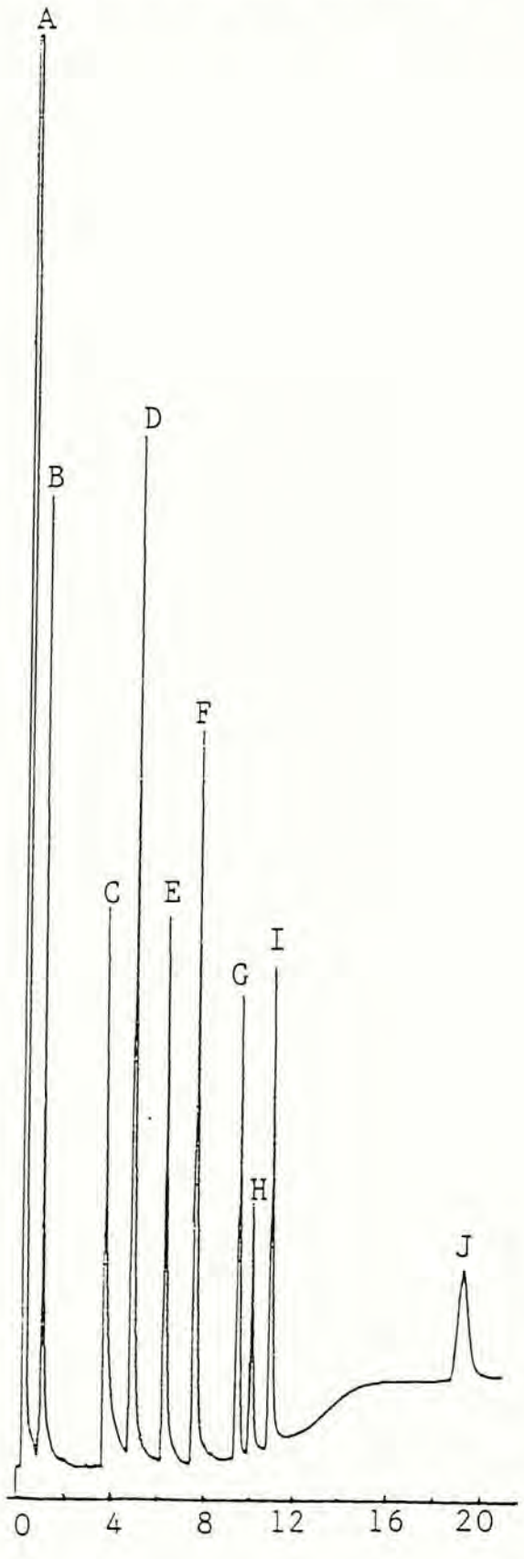
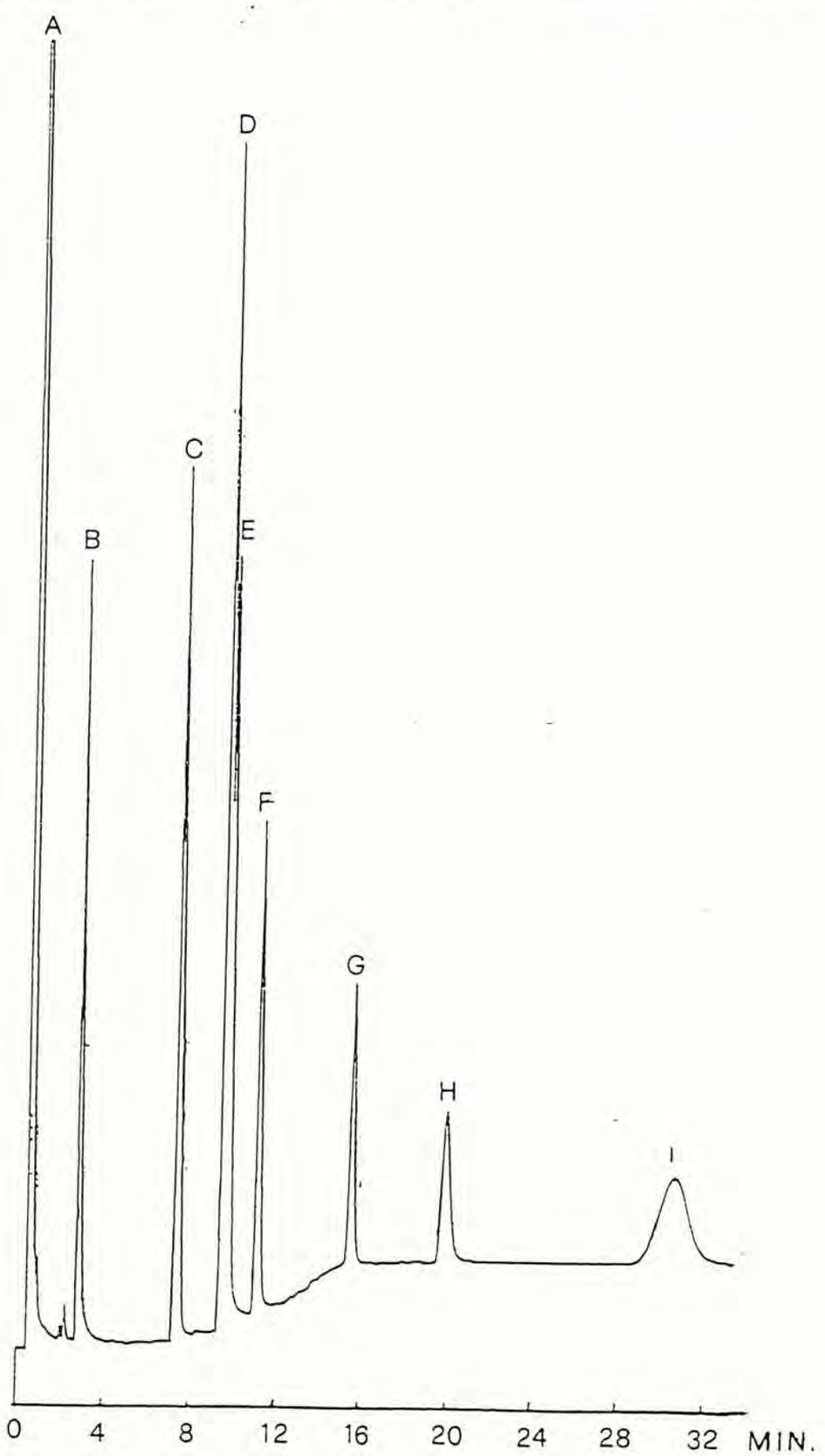


**Figure 2.3.2-4** Gas chromatograms of the drugs of interest obtained by a column of 3% **OV-225** under the optimised conditions. A, solvent; B, ephedrine hydrochloride (2.98 mg/ml); C, guaiphenesin (5.95 mg/ml); D, diphenhydramine hydrochloride (2.50 mg/ml); E, chlorpheniramine maleate (3.03 mg/ml); F, dextromethorphan hydrobromide (1.93 mg/ml); G, bromhexine hydrochloride (1.96 mg/ml), H, codeine phosphate (1.93 mg/ml); and I, papaverine hydrochloride (2.99 mg/ml).



### 2.3.3 Optimization of The Instrumental Parameters

In gas chromatography, there are several instrumental parameters needed to be optimised, including the flow rate of carrier gas, flow rates of hydrogen and air for the FID detector, and temperature programming conditions. The flow rate of carrier gas, nitrogen, was chosen to be 30 ml/min., which was not uncommon in analysis of drugs. The flow rates of hydrogen and air were suggested to be 30 and 300 ml/min., respectively[13]. The optimization of temperature programming conditions is so important because it may affect the overall running time, resolution of the peaks, and sensitivities of the signals.

In the previous section, the rough temperature programming conditions for various columns were found by observing the resolution of the peaks and overall running times of the chromatograms. After choosing the best stationary phase, the temperature programming conditions were then optimised.

An aqueous standard solution containing known amounts of bromhexine hydrochloride, chlorpheniramine maleate, codeine phosphate, dextromethorphan hydrobromide, diphenhydramine hydrochloride, ephedrine hydrochloride, guaiphenesin, and papaverine hydrochloride was prepared. An aliquot (10 ml) of the aqueous standard solution was extracted into chloroform by the procedure mentioned in the Experimental section, and a series of standard solution for injection was then prepared. The final temperature of the temperature programming was chosen to be 265 °C because a higher temperature could shorten the life of the column while a lower temperature would lengthen the overall running time. By fixing the heating rate and final temperature of the temperature programming, the initial temperature was optimised. For each initial temperature, calibration graph of each drug was obtained, and the results are listed in Table 2.3.3-1. It was found that the slopes of the calibration graphs

(or the sensitivities) for the drugs slightly increased with increasing the initial temperature. However, the peak of ephedrine (or pseudoephedrine) was very close to the solvent peak at an initial temperature of 190 °C. Finally, an initial temperature of 170 °C was chosen, which offered a better sensitivity for each drug.

After choosing the initial temperature, the heating rate was adjusted. By fixing the initial and final temperature of the temperature programming, retention times of the drugs at various heating rates were obtained. The results are shown in Table 2.3.3-2. The overall running time decreased with increasing the heating rate. However, the faster the heating rate, the poorer was the resolution of the peaks. In order to have a faster running time and better resolution of the peaks, a heating rate of 10 °C/min. was selected.

In order to test the performance (or reproducibility) of the optimised temperature programming, a chloroform solution containing all the drugs under study was prepared and injected ten times onto the column under the optimised chromatographic conditions, and the relative standard deviation of the peak area ratios for each drug was then calculated. The results are shown in Table 2.3.3-3. The relative standard deviation of each drug ranged from 0.49 to 4.7%, indicating that the reproducibility of the optimised temperature programming was good enough.

**Table 2.3.3-1** The slopes of the calibration graphs of the drugs at various initial temperatures but with fixed heating rate and final temperature of the temperature programming[+].

| Compounds | Slopes of the calibration graphs, ml/mg | | |
|---|---|---|---|
| | Initial temperature[*], °C | | |
| | 150 | 170 | 190 |
| Bromhexine HCl | 2.137 | 2.139 | 2.210 |
| Chlorpheniramine maleate | 2.358 | 2.544 | 2.566 |
| Codeine phosphate | 2.149 | 2.188 | 2.237 |
| Dextromethorphan HBr | 3.098 | 3.177 | 2.998 |
| Diphenhydramine HCl | 3.439 | 3.576 | 3.929 |
| Ephedrine HCl | 2.428 | 2.953 | n.d.[**] |
| Guaiphenesin | 1.929 | 1.994 | 2.215 |
| Papaverine HCl | 2.926 | 3.125 | 3.169 |

[+]The heating rate was set at 10 °C/min.; and the final temperature was set at 265 °C and kept for 10 min.

[*]The initial temperature was kept for 1 min.

[**]The peak of ephedrine was too close to the solvent peak and therefore no calibration graph was plotted.

**Table 2.3.3-2** Retention times of the drugs at various heating rates but with fixed initial and final temperatures for the temperature programming[+].

| Compounds | Retention times, min. | | |
|---|---|---|---|
| | Heating rate, °C/min. | | |
| | 8 | 10 | 12 |
| Ephedrine HCl | 1.06 | 1.05 | 1.05 |
| Guaiphenesin | 3.79 | 3.64 | 3.54 |
| Diphenhydramine HCl | 5.15 | 4.90 | 4.62 |
| Chlorpheniramine maleate | 6.70 | 6.24 | 5.80 |
| Dextromethorphan HBr | 8.21 | 7.52 | 6.90 |
| Bromhexine HCl | 10.48 | 9.37 | 8.50 |
| Codeine phosphate | 12.26 | 10.85 | 9.76 |
| Papaverine HCl | 20.80 | 19.12 | 17.82 |

[+]The initial temperature was set at 170 °C and kept for 1 min.; and the final temperature was set at 265 °C and kept for 10 min.

**Table 2.3.3-3** Data for testing the reproducibility of the optimised temperature programming.

| Drug | Concentration mg/ml | Relative standard deviation* (%) |
|---|---|---|
| Bromhexine HCl | 0.7720 | 0.73 |
| Chlorpheniramine maleate | 0.9160 | 1.3 |
| Codeine phosphate | 0.9120 | 2.0 |
| Dextromethorphan HBr | 1.004 | 0.49 |
| Diphenhydramine HCl | 1.092 | 1.3 |
| Ephedrine HCl | 0.8680 | 4.7 |
| Guaiphenesin | 1.536 | 3.4 |
| Papaverine HCl | 0.4680 | 3.3 |
| Pseudoephedrine HCl | 1.250 | 1.7 |

*For ten replicate injections.

2.3.4 Preparation of The Calibration Graphs

The calibration graph of each drug under study was obtained by plotting the peak area ratios (drug to internal standard) against the corresponding injection concentrations. In quantitative determination of the drugs in real samples, results were deduced from the respective calibration graph for each drug with the help of the linear regression equation.

2.3.4.1 *Calibration graph of bromhexine hydrochloride*

An aqueous standard solution of bromhexine hydrochloride (0.3016 mg/ml) was prepared by dissolving appropriate amount of the drug in 50 ml of sulphuric acid (0.1 M) in a calibrated flask. 10 ml of the aqueous standard solution was made alkaline with ammonia solution and extracted with chloroform (3 x 15 ml), detailed procedure being found in the Experimental section. The organic layer (chloroform) was filtered after being dried with anhydrous sodium sulphate, and the filtrate was made up to 50 ml with chloroform in a calibrated flasks (Solution I). 15.00, 12.50, 10.00, 8.00, 6.00, 4.00, 2.00, and 1.00 ml of Solution I were pipetted separately into a series of V-flasks and evaporated to dryness under nitrogen in a water bath at room temperature. The residue was completely dissolved in exactly 1 ml of the internal standard solution (Solution II), 3.0 $\mu$l of which was injected onto the chromatograph under the chromatographic conditions described in the Experimental section. All injection was performed in duplicate. The calibration graph was obtained by plotting the peak area ratios (drug to internal standard) against concentrations of Solution II. The data of the calibration graph are listed in Table 2.3.4.1-1, and the calibration graph is shown in Figure 2.3.4.1-1.

**Table 2.3.4.1-1** Data for the calibration graph of bromhexine hydrochloride.

| Concentration of bromhexine hydrochloride, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.06032 | 0.1395 |
| 0.1206 | 0.2877 |
| 0.2413 | 0.5876 |
| 0.3619 | 0.9348 |
| 0.4826 | 1.252 |
| 0.6032 | 1.579 |
| 0.7540 | 1.967 |
| 0.9048 | 2.378 |

Linear working range = 0.06 - 0.90 mg/ml;

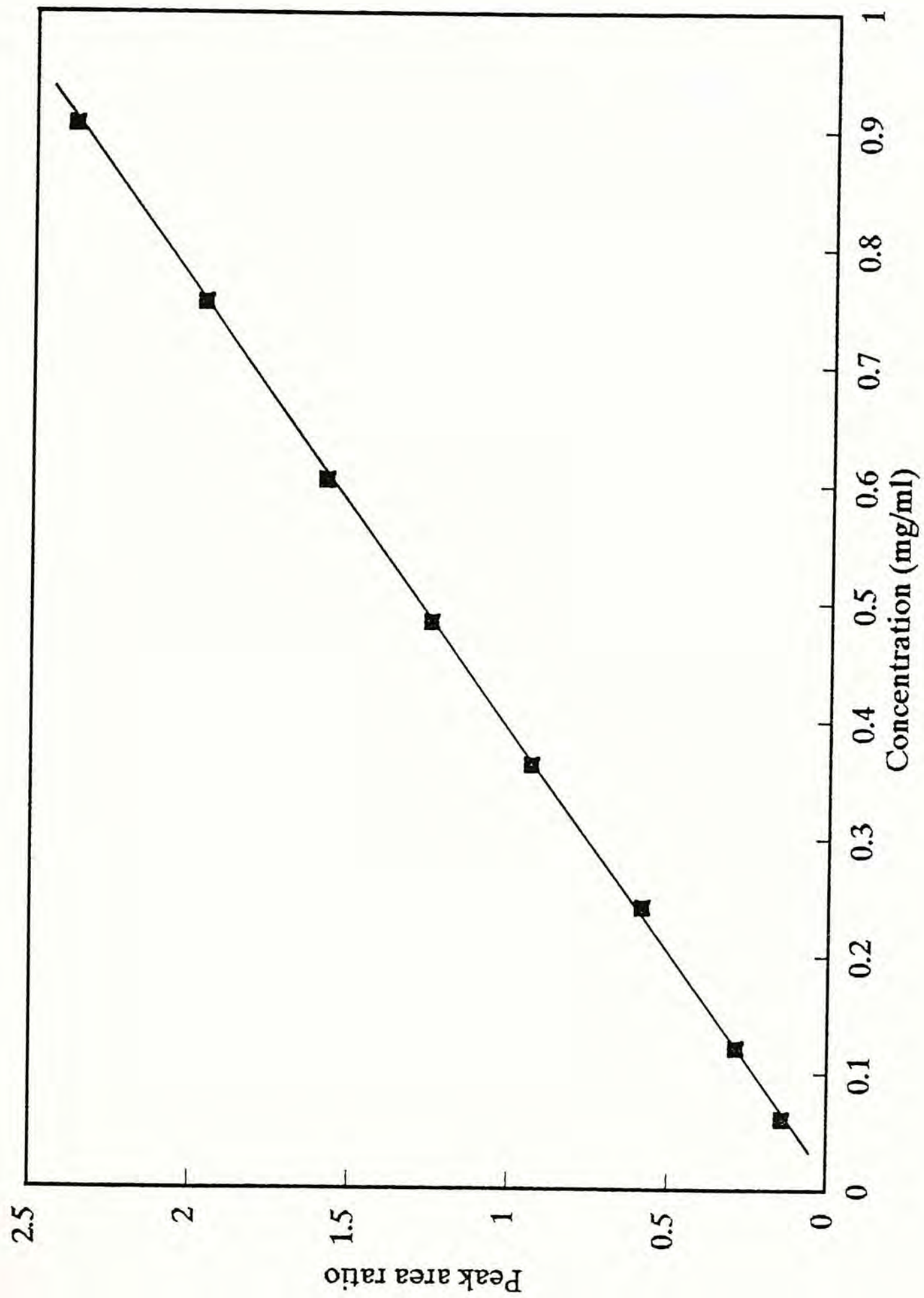
Slope = 2.661 ml/mg;

Intercept = -0.03312;

Correlation coefficient = 0.9999; and

Detectable amount per injection = 0.18 $\mu$g.

**Figure 2.3.4.1-1** Calibration graph of bromhexine hydrochloride.



2.3.4.2 *Calibration graph of chlorpheniramine maleate*

The calibration graph of chlorpheniramine maleate was prepared using an aqueous standard solution of chlorpheniramine maleate (0.3008 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are listed in Table 2.3.4.2-1, and the calibration graph is shown in Figure 2.3.4.2-1.

**Table 2.3.4.2-1** Data for the calibration graph of chlorpheniramine maleate.

| Concentration of chlorpheniramine maleate, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.06016 | 0.07811 |
| 0.1203 | 0.2166 |
| 0.2406 | 0.5179 |
| 0.3610 | 0.8307 |
| 0.4813 | 1.154 |
| 0.6016 | 1.480 |
| 0.7520 | 1.911 |
| 0.9024 | 2.293 |

Linear working range = 0.06 - 0.90 mg/ml;

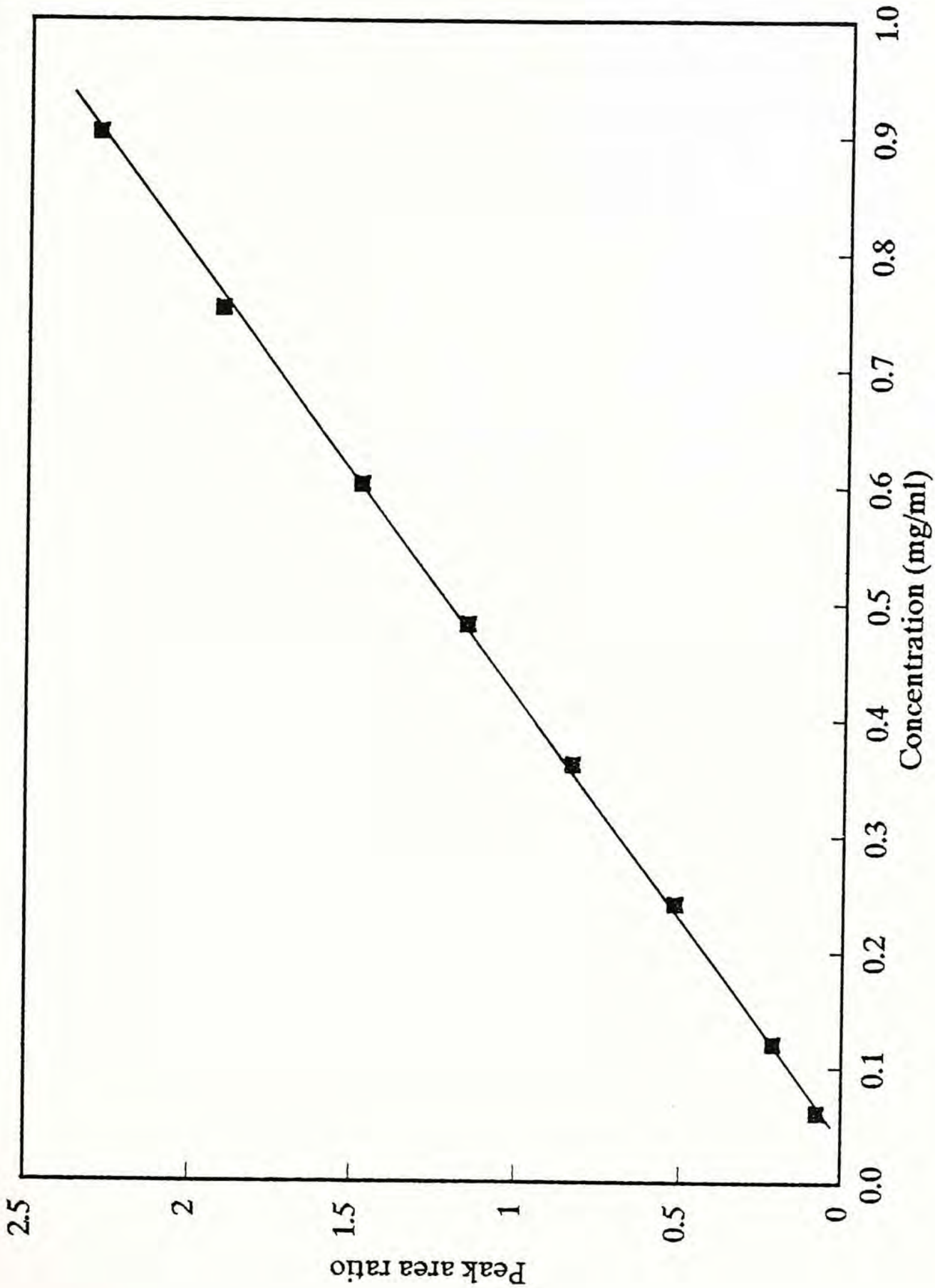
Slope = 2.654 ml/mg;

Intercept = -0.1076;

Correlation coefficient = 0.9997; and

Detectable amount per injection = 0.18 $\mu$g.

**Figure 2.3.4.2-1** Calibration graph of chlorpheniramine maleate.



2.3.4.3 *Calibration graph of codeine phosphate*

The calibration graph of codeine phosphate was prepared using an aqueous standard solution of codeine phosphate (0.5128 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are listed in Table 2.3.4.3-1, and the calibration graph is shown in Figure 2.3.4.3-1.

**Table 2.3.4.3-1** Data for the calibration graph of codeine phosphate.

| Concentration of codeine phosphate, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.1026 | 0.1865 |
| 0.2051 | 0.4527 |
| 0.4102 | 0.9994 |
| 0.6154 | 1.589 |
| 0.8205 | 2.161 |
| 1.026 | 2.734 |
| 1.282 | 3.468 |
| 1.538 | 4.218 |

Linear working range = 0.10 - 1.54 mg/ml;

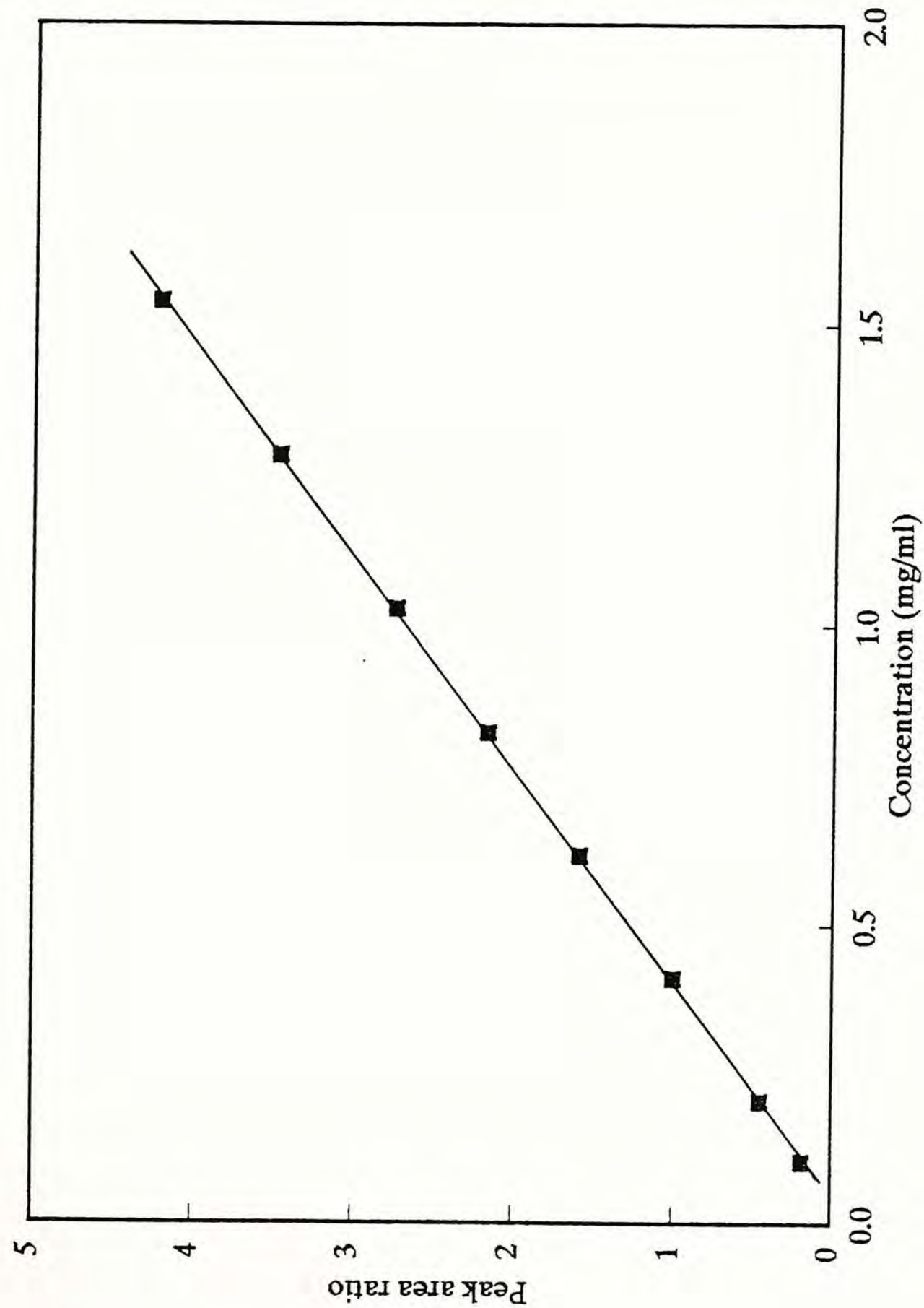
Slope = 2.810 ml/mg;

Intercept = -0.1312;

Correlation coefficient = 0.9999; and

Detectable amount per injection = 0.30 $\mu$g.

**Figure 2.3.4.3-1** Calibration graph of codeine phosphate.



2.3.4.4 *Calibration graph of dextromethorphan hydrobromide*

The calibration graph of dextromethorphan hydrobromide was prepared using an aqueous standard solution of dextromethorphan hydrobromide (0.5056 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are listed in Table 2.3.4.4-1, and the calibration graph is shown in Figure 2.3.4.4-1.

**Table 2.3.4.4-1** Data for the calibration graph of dextromethorphan hydrobromide.

| Concentration of dextromethorphan hydrobromide, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.2022 | 0.3598 |
| 0.4045 | 1.087 |
| 0.6067 | 1.764 |
| 0.8090 | 2.462 |
| 1.011 | 3.206 |
| 1.264 | 4.059 |
| 1.517 | 4.926 |

Linear working range = 0.20 - 1.52 mg/ml;

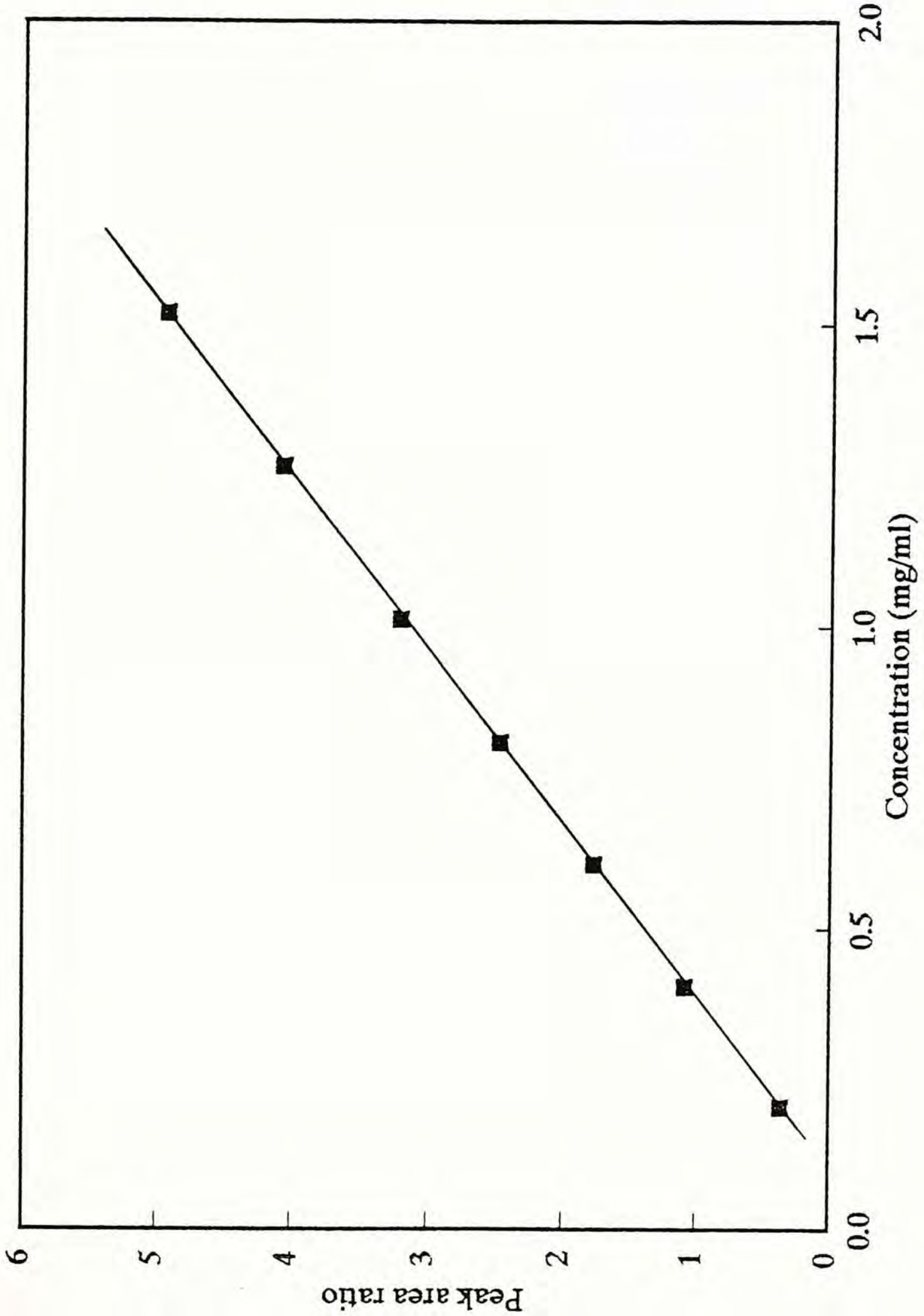
Slope = 3.474 ml/mg;

Intercept = -0.3337;

Correlation coefficient = 0.9999; and

Detectable amount per injection = 0.60 $\mu$g.

**Figure 2.3.4.4-1** Calibration graph of dextromethorphan hydrobromide.



2.3.4.5 *Calibration graph of diphenhydramine hydrochloride*

The calibration graph of diphenhydramine hydrochloride was prepared using an aqueous standard solution of diphenhydramine hydrochloride (0.5248 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are listed in Table 2.3.4.5-1, and the calibration graph is shown in Figure 2.3.4.5-1.

**Table 2.3.4.5-1** Data for the calibration graph of diphenhydramine hydrochloride.

| Concentration of diphenhydramine hydrochloride, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.2099 | 0.5227 |
| 0.4198 | 1.395 |
| 0.6298 | 2.307 |
| 0.8397 | 3.240 |
| 1.050 | 4.150 |
| 1.312 | 5.282 |
| 1.575 | 6.383 |

Linear working range = 0.20 - 1.58 mg/ml;

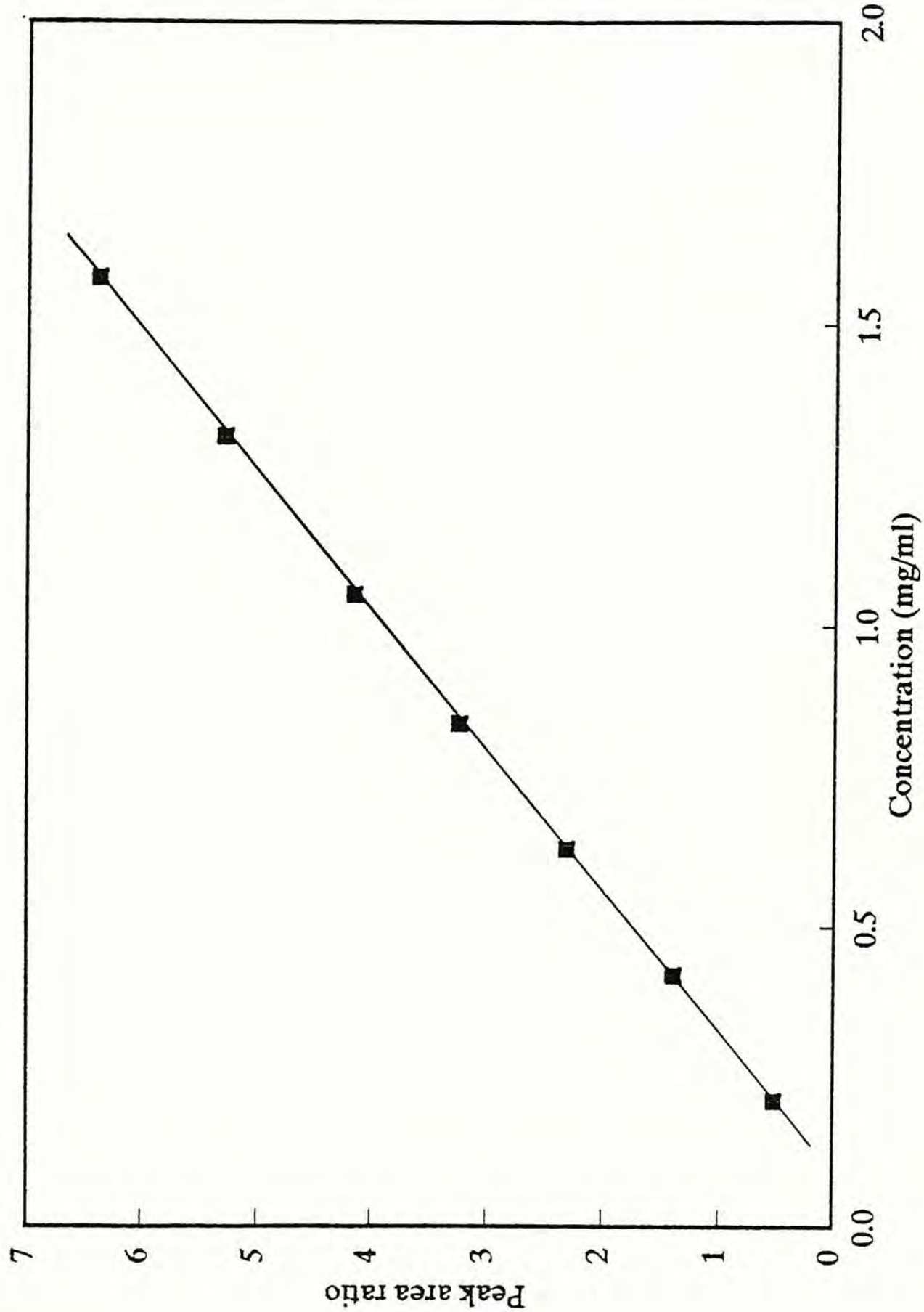
Slope = 4.316 ml/mg;

Intercept = -0.3963;

Correlation coefficient = 0.9999; and

Detectable amount per injection = 0.60 $\mu$g.

**Figure 2.3.4.5-1** Calibration graph of diphenhydramine hydrochloride.



2.3.4.6 *Calibration graph of ephedrine hydrochloride*

The calibration graph of ephedrine hydrochloride was prepared using an aqueous standard solution of ephedrine hydrochloride (0.5120 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are listed in Table 2.3.4.6-1, and the calibration graph is shown in Figure 2.3.4.6-1. The calibration graph exhibited a large negative intercept and showed a working range between 0.4 and 1.54 mg/ml. There was no detectable signal of ephedrine at a concentration below 0.4 mg/ml. In comparison with other drugs under study, ephedrine was a volatile compound, and part of it would probably be lost during evaporation under nitrogen. The loss of ephedrine in evaporation became significant at low concentration of the compound, and hence, no signal was detected at a concentration below 0.4 mg/ml.

**Table 2.3.4.6-1** Data for the calibration graph of ephedrine hydrochloride.

| Concentration of ephedrine hydrochloride, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.4096 | 0.1111 |
| 0.6144 | 0.7289 |
| 0.8192 | 1.357 |
| 1.024 | 1.976 |
| 1.280 | 2.816 |
| 1.536 | 3.587 |

Linear working range = 0.40 - 1.54 mg/ml;

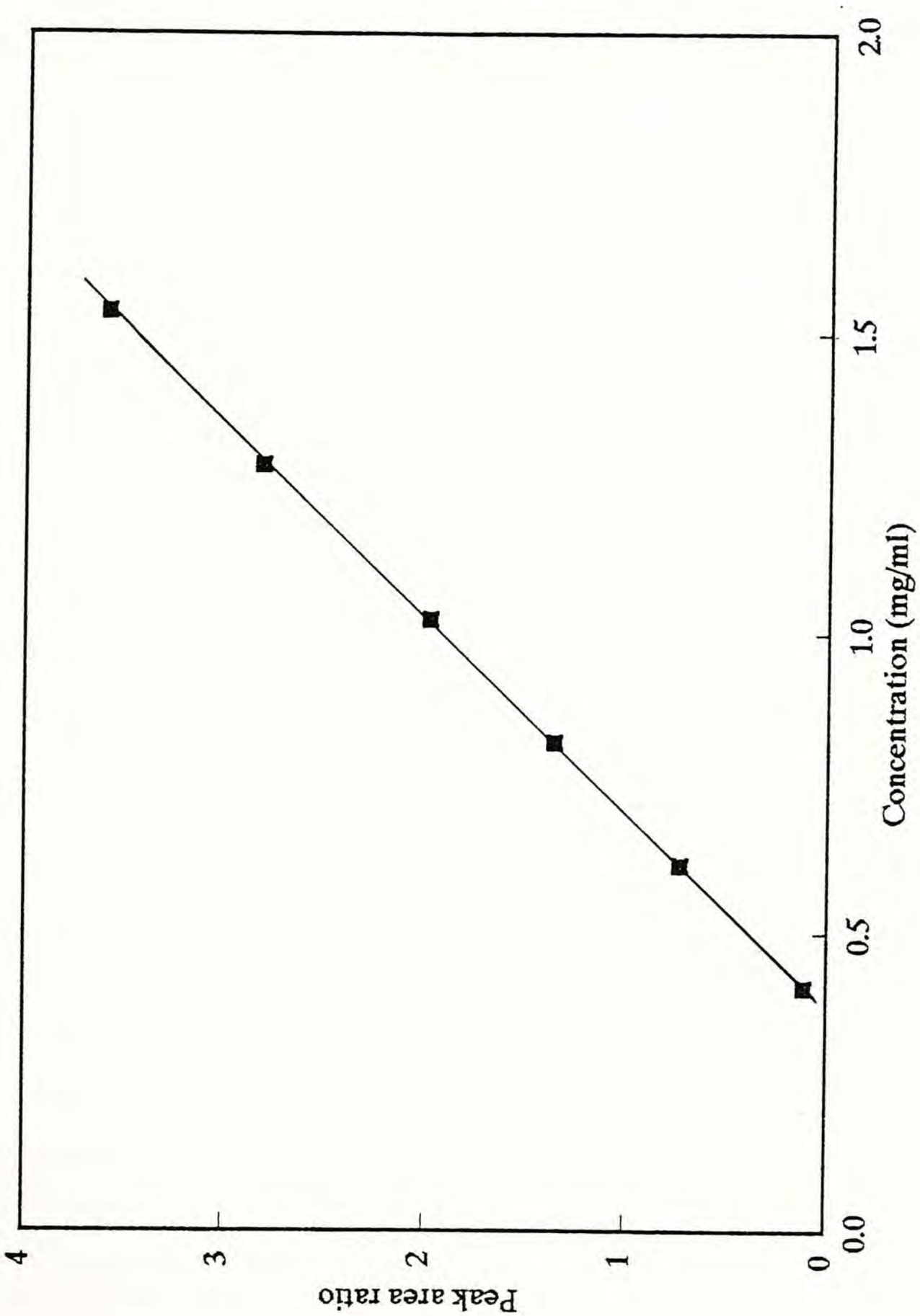
Slope = 3.098 ml/mg;

Intercept = -1.172;

Correlation coefficient = 0.99992; and

Detectable amount per injection = 1.20 $\mu$g.

**Figure 2.3.4.6-1** Calibration graph of ephedrine hydrochloride.



2.3.4.7 *Calibration graph of guaiphenesin*

The calibration graph of guaiphenesin was prepared using an aqueous standard solution of guaiphenesin (1.795 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are listed in Table 2.3.4.7-1, and the calibration graph is shown in Figure 2.3.4.7-1.

**Table 2.3.4.7-1** Data for the calibration graph of guaiphenesin.

| Concentration of guaiphenesin, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.3589 | 0.5156 |
| 0.7178 | 1.415 |
| 1.436 | 3.402 |
| 2.153 | 5.555 |
| 2.871 | 7.562 |
| 3.589 | 9.818 |
| 4.486 | 12.43 |
| 5.384 | 15.03 |

Linear working range = 0.35 - 5.38 mg/ml;

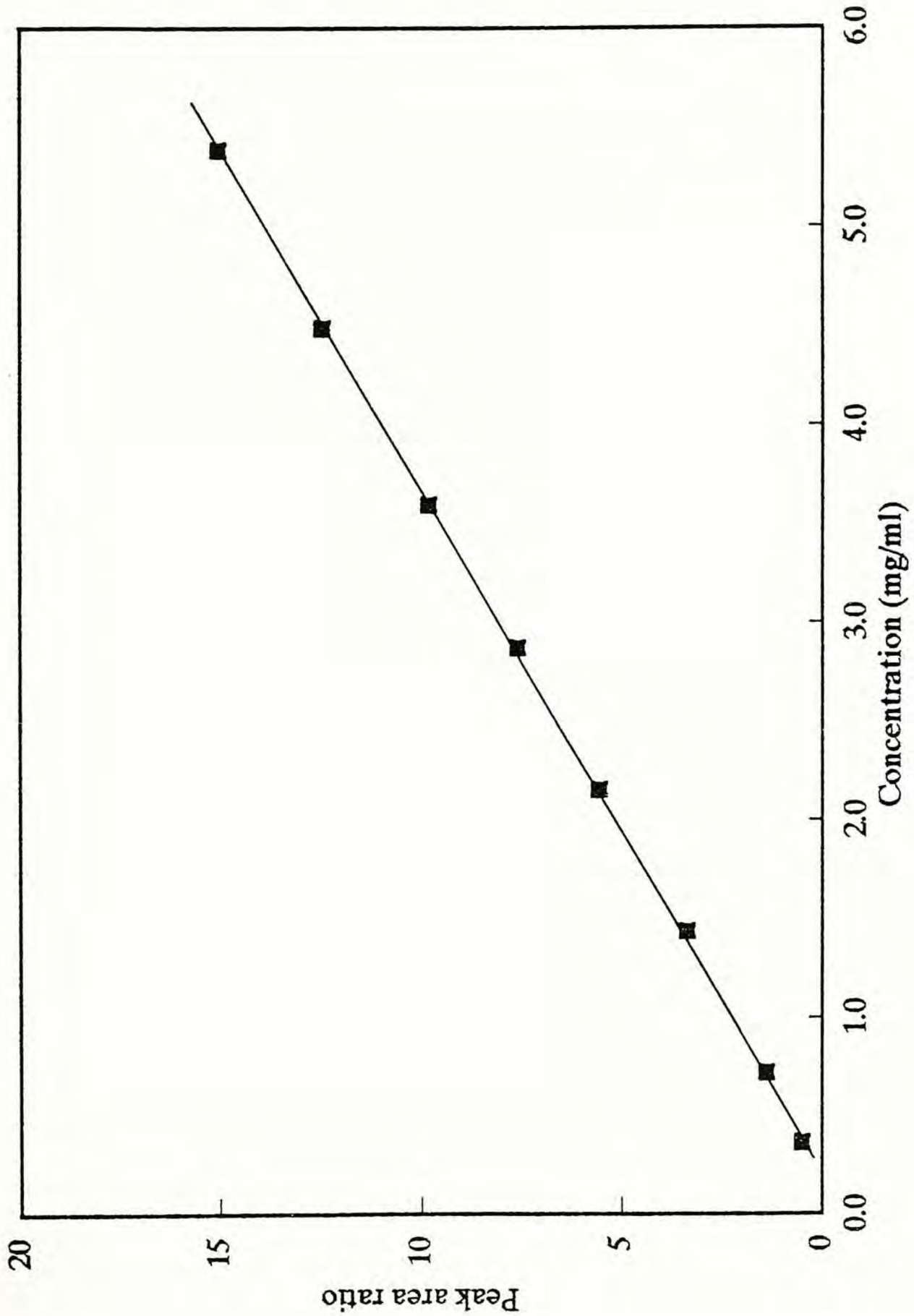
Slope = 2.911 ml/mg;

Intercept = -0.6736;

Correlation coefficient = 0.9998; and

Detectable amount per injection = 1.10 $\mu$g.

**Figure 2.3.4.7-1** Calibration graph of guaiphenesin.



2.3.4.8 *Calibration graph of papaverine hydrochloride*

The calibration graph of papaverine hydrochloride was prepared using an aqueous standard solution of papaverine hydrochloride (0.1576 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are shown in Table 2.3.4.8-1, and the calibration graph is shown in Figure 2.3.4.8-1.

**Table 2.3.4.8-1** Data for the calibration graph of papaverine hydrochloride.

| Concentration of papaverine hydrochloride, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.03152 | 0.06256 |
| 0.06304 | 0.1459 |
| 0.1261 | 0.3509 |
| 0.1891 | 0.5637 |
| 0.2522 | 0.7593 |
| 0.3152 | 0.9809 |
| 0.3940 | 1.225 |
| 0.4728 | 1.468 |

Linear working range = 0.03 - 0.48 mg/ml;

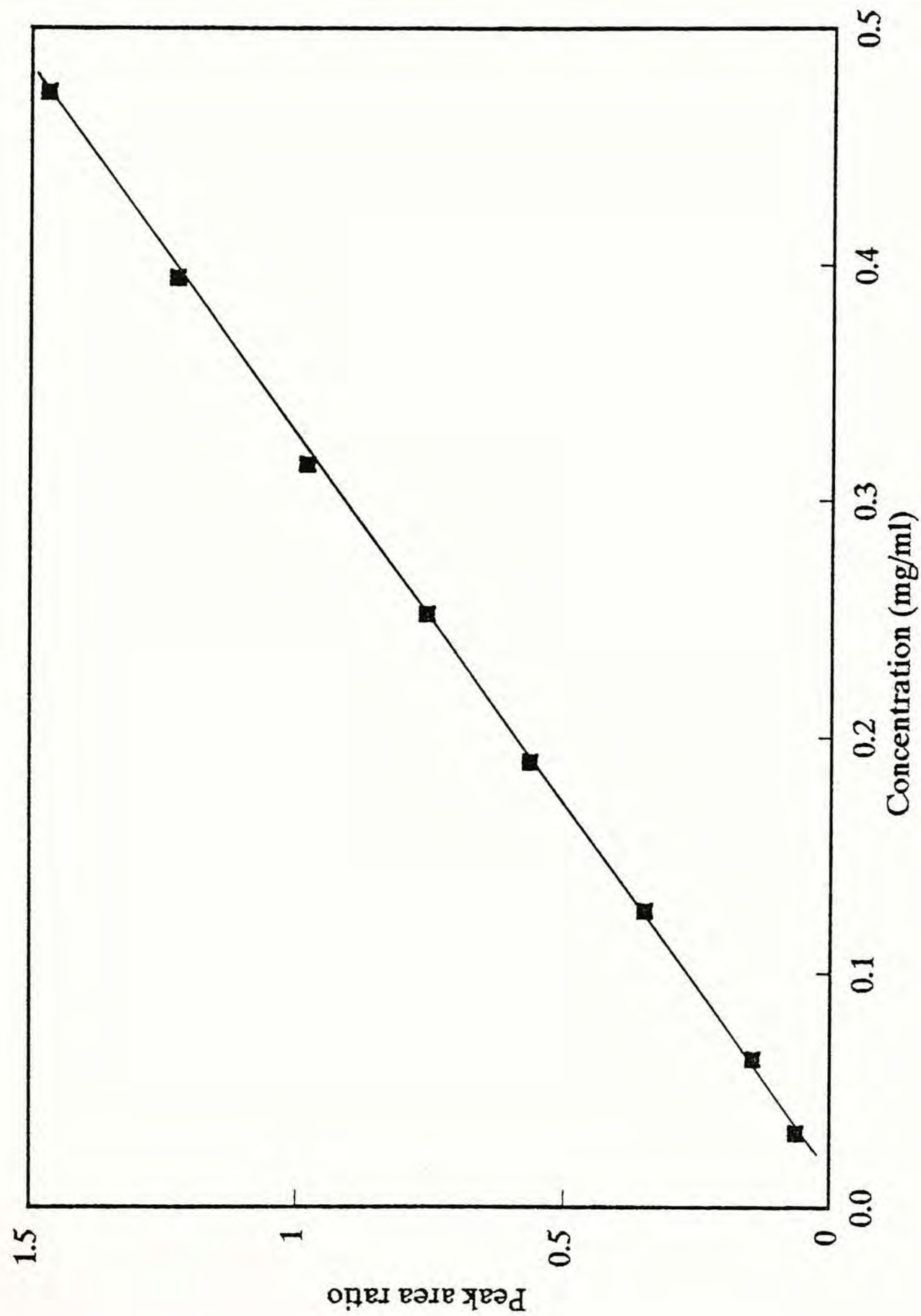
Slope = 3.223 ml/mg;

Intercept = -0.04843;

Correlation coefficient = 0.9998; and

Detectable amount per injection = 0.10 $\mu$g.

**Figure 2.3.4.8-1** Calibration graph of papaverine hydrochloride.



2.3.4.9 *Calibration graph of pseudoephedrine hydrochloride*

The calibration graph of pseudoephedrine hydrochloride was prepared using an aqueous standard solution of pseudoephedrine hydrochloride (0.6240 mg/ml) and following the procedure for bromhexine hydrochloride. The data of the calibration graph are listed in Table 2.3.4.9-1, and the calibration graph is shown in Figure 2.3.4.9-1. The calibration graph of pseudoephedrine was similar to that of ephedrine, which also showed a large negative intercept. In fact, no signal of pseudoephedrine was detected at a concentration below 0.49 mg/ml. Same as ephedrine, the absence of signals of pseudoephedrine at low concentration might be due to the loss of the compound during evaporation under nitrogen.

**Table 2.3.4.9-1** Data for the calibration graph of pseudoephedrine hydrochloride.

| Concentration of pseudoephedrine hydrochloride, mg/ml | Mean peak area ratio (n=2) |
|---|---|
| 0.4992 | 0.5137 |
| 0.7488 | 1.193 |
| 0.9984 | 1.905 |
| 1.248 | 2.631 |
| 1.560 | 3.465 |
| 1.872 | 4.334 |

Linear working range = 0.50 - 1.87 mg/ml;

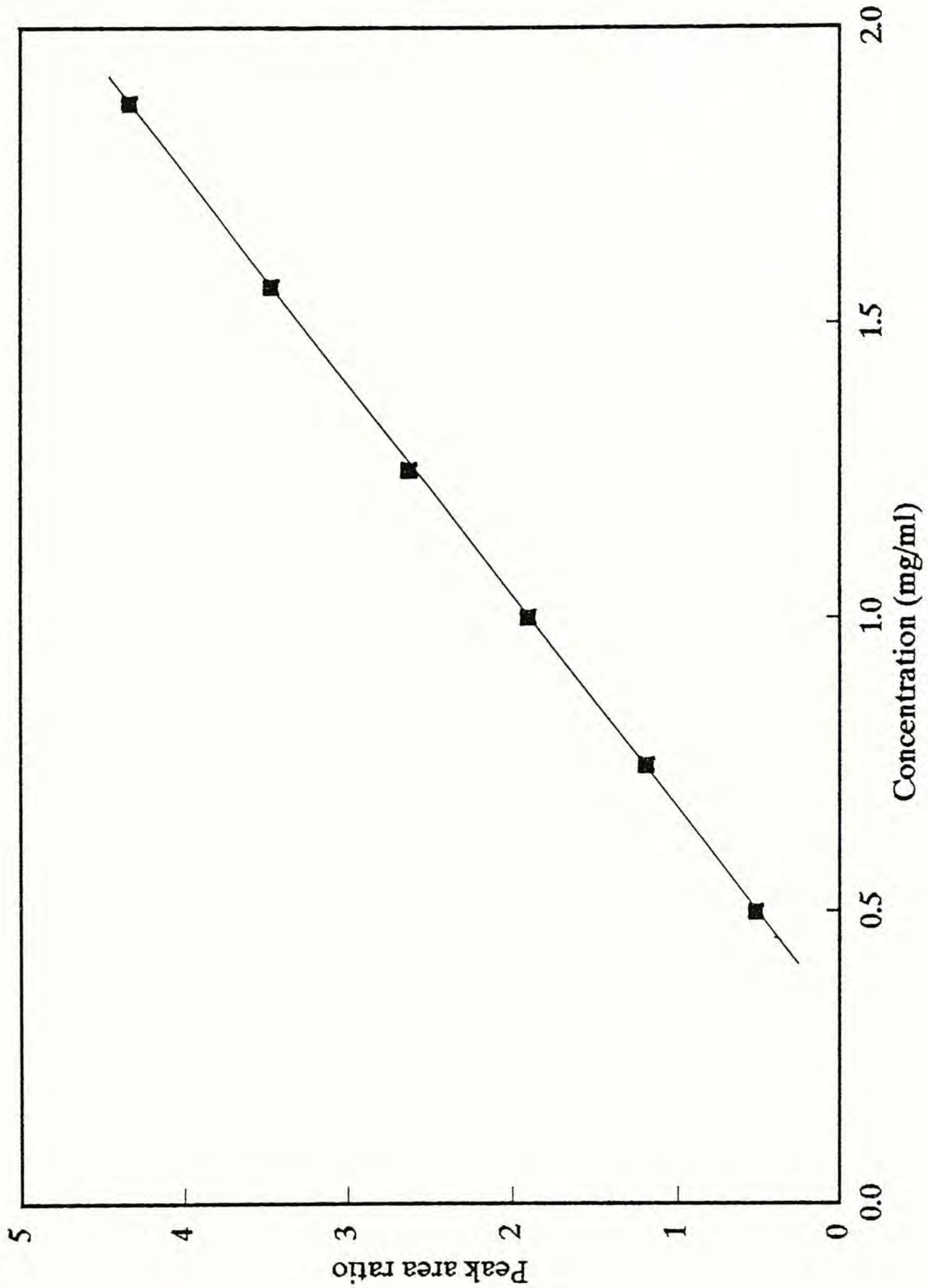
Slope = 2.790 ml/mg;

Intercept = -0.8799;

Correlation coefficient = 0.9999; and

Detectable amount per injection = 1.50 $\mu$g.

**Figure 2.3.4.9-1** Calibration graph of pseudoephedrine hydrochloride.



2.3.5 Recovery Test and Precision

In order to test the reliability of the proposed method, recovery tests were performed by adding known amount of each drug to a cough-cold syrup where it was known to be absent. For each drug, four replicate determinations were made.

As an example, the recovery tests of bromhexine hydrochloride, guaiphenesin and pseudoephedrine hydrochloride were performed with the following procedure:

An aqueous standard solution with known amounts of bromhexine hydrochloride (1.508 mg/ml), guaiphenesin (8.972 mg/ml) and pseudoephedrine hydrochloride (3.120 mg/ml) was prepared. 10 ml of the standard solution was pipetted into a 25-ml V-flask and diluted to mark with a cough-cold syrup where bromhexine hydrochloride, guaiphenesin and pseudoephedrine hydrochloride were absent (Sample I). The amounts of the added drugs in Sample I were determined using the proposed method as described in the Experimental section. The recovery of each drug was then calculated. The recovery tests of other drugs under study were performed in a similar manner. The results are present in Table 2.3.5-1. The mean results of four analyses for each drug ranged from 96.0 to 99.7%, which can be considered to be good recoveries.

The precision of the proposed method was checked by calculating the relative standard deviation for the above determinations, and the results are also shown in Table 2.3.5-1. The relative standard deviation of four analyses for each drug under study ranged from 0.52 to 1.77%.

**Table 2.3.5-1** Results of the recovery tests and precision for the drugs under study using the proposed method.

| Drug | Amount added (mg/5 ml) | Recovery* (%) | Mean recovery (%) | Relative standard deviation (%) |
|---|---|---|---|---|
| Bromhexine HCl | 3.016 | 95.43<br>95.78<br>96.78<br>96.63 | 96.2 | 0.59 |
| Chlorpheniramine maleate | 3.008 | 98.10<br>99.60<br>96.32<br>97.43 | 97.9 | 1.21 |
| Codeine phosphate | 5.015 | 99.69<br>99.68<br>101.5<br>98.09 | 99.7 | 1.21 |
| Dextromethorphan HBr | 5.080 | 96.19<br>96.72<br>95.81<br>95.37 | 96.0 | 0.52 |
| Diphenhydramine HCl | 5.250 | 99.66<br>97.32<br>100.1<br>98.04 | 98.8 | 1.15 |
| Ephedrine HCl | 5.040 | 98.00<br>99.10<br>96.61<br>97.39 | 97.8 | 0.93 |
| Guaiphenesin | 17.95 | 98.77<br>99.72<br>96.19<br>97.80 | 98.1 | 1.33 |

cont'd **Table 2.3.5-1**

| Drug | Amount added (mg/5 ml) | Recovery* (%) | Mean recovery (%) | Relative standard deviation (%) |
|---|---|---|---|---|
| Papaverine HCl | 1.680 | 94.51<br>98.21<br>96.66<br>98.45 | 97.0 | 1.62 |
| Pseudoephedrine HCl | 6.24 | 95.94<br>98.68<br>95.64<br>99.64 | 97.5 | 1.77 |

2.3.6 Determination of active ingredients in cough-cold syrups

The contents of the drugs under study in nine commercially available cough-cold syrups were determined in triplicate using the proposed method. The samples under study contained combinations of several active ingredients, which were identified by comparing the retention time of the peaks observed with those obtained from standard solutions containing the respective active ingredients examined under the same conditions. The results obtained were compared with those obtained using an established HPLC method[3] and with the corresponding label values. A brief description of the samples is shown in Table 2.3.6-1, and the results of the sample analysis are present in Table 2.3.6-2. Typical chromatograms for sample No.1 and No.9 are shown in Figure 2.3.6-1 and Figure 2.3.6-2, respectively, where no extra peaks due to other excipients are evident.

From Table 2.3.6-1, it can be seen that the results obtained with the proposed method were in good agreement with those obtained using the counter-check HPLC method and with the respective label values.

**Table 2.3.6-1** Brief description of the samples.

| Batch No. | Name | Source | Ingredients | Labelled amount (mg/5ml) |
|---|---|---|---|---|
| 1 | DECOXINE Cough Syrup | Commercially available | Bromhexine HCl | 2.5 |
| | | | Codeine phosphate | 4.5 |
| | | | Diphenhydramine HCl | 4.5 |
| | | | Ephedrine HCl | 4.5 |
| | | | Papaverine HCl | 1.25 |
| 2 | COLDREX Cough Syrup | Commercially available | Chlorpheniramine maleate | 1.0 |
| | | | Dextromethorphan HBr | 5.0 |
| | | | Guaiphenesin | 25.0 |
| | | | Phenylephrine HCl | 5.0 |
| 3 | BREACOL Cough Syrup | Commercially available | Chlorpheniramine maleate | 1.0 |
| | | | Dextromethorphan HBr | 5.0 |
| | | | Guaiphenesin | 25.0 |
| | | | Menthol | 5.0 |
| | | | Phenylephrine HCl | 5.0 |
| 4 | P.E.C. Syrup | Universal Pharmaceutical Laboratories, Ltd., H.K. | Codeine phosphate | 9.0 |
| | | | Ephedrine HCl | 7.2 |
| | | | Promethazine HCl | 3.6 |
| | | | Flavoured vehicle | q.s. |
| 5 | UNI-VASIN LIQUID | Universal Pharmaceutical Laboratories, Ltd., H.K. | Chlorpheniramine maleate | 4.0 |
| | | | Phenylephrine HCl | 5.0 |
| | | | Phenylpropanolamine HCl | 5.0 |
| | | | Flavoured vehicle | q.s. |

cont'd **Table 2.3.6-1.**

| Batch No. | Name | Source | Ingredients | Labelled amount (mg/5ml) |
|---|---|---|---|---|
| 6 | WANI Cough Syrup | Wah Ning Dispensary Ltd. | Ammonium chloride<br>Chlorpheniramine maleate<br>Codeine phosphate<br>Ephedrine HCl<br>Flavoured syrup | 110<br>2.0<br>10.0<br>5.0<br>q.s. |
| 7 | ROBITUSSIN Cough Syrup | Commercially available | Guaiphenesin<br>Alcohol | 100.0<br>3.5% |
| 8 | SUDAFED Expectorant | Commercially available | Guaiphenesin<br>Pseudoephedrine HCl | 100.0<br>30.0 |
| 9 | ROBITUSSIN Expectorant | Commercially available | Guaiphenesin<br>Pseudoephedrine HCl<br>Alcohol | 100.0<br>30.0<br>1.4% |

**Table 2.3.6-2** The assay results for the determination of various active ingredients in cough-cold syrups.

| Batch No. | Ingredients | Labelled values (mg/5ml) | Percentage of label claim | | |
|---|---|---|---|---|---|
| | | | By proposed method | | By HPLC method |
| | | | amount found | mean* | |
| 1 | Bromhexine HCl | 2.5 | 92.4<br>92.4<br>92.8 | 92.5 (0.2) | 95.2 |
| | Codeine phosphate | 4.5 | 101.3<br>101.3<br>101.8 | 101.5 (0.2) | 103.8 |
| | Diphenhydramine HCl | 4.5 | 104.2<br>104.0<br>104.9 | 104.4 (0.4) | 96.2 |
| | Ephedrine HCl | 4.5 | 101.1<br>100.0<br>103.1 | 101.4 (1.3) | 97.1 |
| | Papaverine HCl | 1.25 | 85.6<br>92.8<br>94.4 | 90.9 (3.8) | 90.4 |
| 2 | Chlorpheniramine maleate | 1.0 | 96.0<br>90.0<br>95.0 | 93.7 (2.6) | 93.0 |
| | Dextromethorphan HBr | 5.0 | 94.8<br>95.6<br>94.8 | 95.3 (0.3) | 99.4 |
| | Guaiphenesin | 25.0 | 96.0<br>98.5<br>95.0 | 96.5 (1.5) | 91.6 |

cont'd **Table 2.3.6-2**

| Batch No. | Ingredients | Labelled values (mg/5ml) | Percentage of label claim | | |
|---|---|---|---|---|---|
| | | | By proposed method | | By HPLC method |
| | | | amount found | mean* | |
| 3 | Chlorpheniramine maleate | 1.0 | 89.2<br>87.3<br>88.5 | 88.3 (0.8) | 93.0 |
| | Dextromethorphan HBr | 5.0 | 94.8<br>95.2<br>95.2 | 95.1 (0.2) | 100.6 |
| | Guaiphenesin | 25.0 | 99.5<br>99.4<br>96.7 | 98.5 (1.3) | 96.4 |
| 4 | Codeine phosphate | 9.0 | 100.0<br>99.2<br>99.4 | 99.6 (0.3) | 102.8 |
| | Ephedrine HCl | 7.2 | 96.2<br>94.4<br>93.8 | 94.8 (1.0) | 97.9 |
| 5 | Chlorpheniramine maleate | 4.0 | 96.9<br>98.1<br>95.9 | 97.0 (0.9) | 102.8 |
| 6 | Chlorpheniramine maleate | 2.0 | 83.5<br>85.0<br>84.8 | 84.4 (0.7) | 85.5 |
| | Codeine phosphate | 10.0 | 86.0<br>92.5<br>93.3 | 90.6 (3.3) | 88.5 |
| | Ephedrine HCl | 7.2 | 86.0<br>94.0<br>90.0 | 90.0 (3.3) | 89.0 |
| 7 | Guaiphenesin | 100.0 | 101.3<br>104.1<br>103.8 | 103.1 (1.3) | 102.5 |

cont'd **Table 2.3.6-2**

| Batch No. | Ingredients | Labelled values (mg/5ml) | Percentage of label claim | | |
|---|---|---|---|---|---|
| | | | By proposed method | | By HPLC method |
| | | | amount found | mean* | |
| 8 | Guaiphenesin | 100.0 | 113.3<br>111.7<br>109.2 | 111.4 (1.7) | 115.3 |
| | Pseudoephedrine HCl | 30.0 | 107.8<br>104.4<br>111.1 | 107.8 (2.7) | 111.9 |
| 9 | Guaiphenesin | 100.0 | 97.4<br>96.6<br>97.9 | 97.3 (0.5) | 99.4 |
| | Pseudoephedrine HCl | 30.0 | 100.9<br>106.1<br>99.9 | 102.3 (2.7) | 105.6 |

*Standard deviation is enclosed in parentheses.

**Figure 2.3.6-1** The chromatogram of sample No.1, with peaks of A, ephedrine hydrochloride; B, diphenhydramine hydrochloride; C, bromhexine hydrochloride; D, clomipramine hydrochloride; E, codeine phosphate; and F, papaverine hydrochloride.

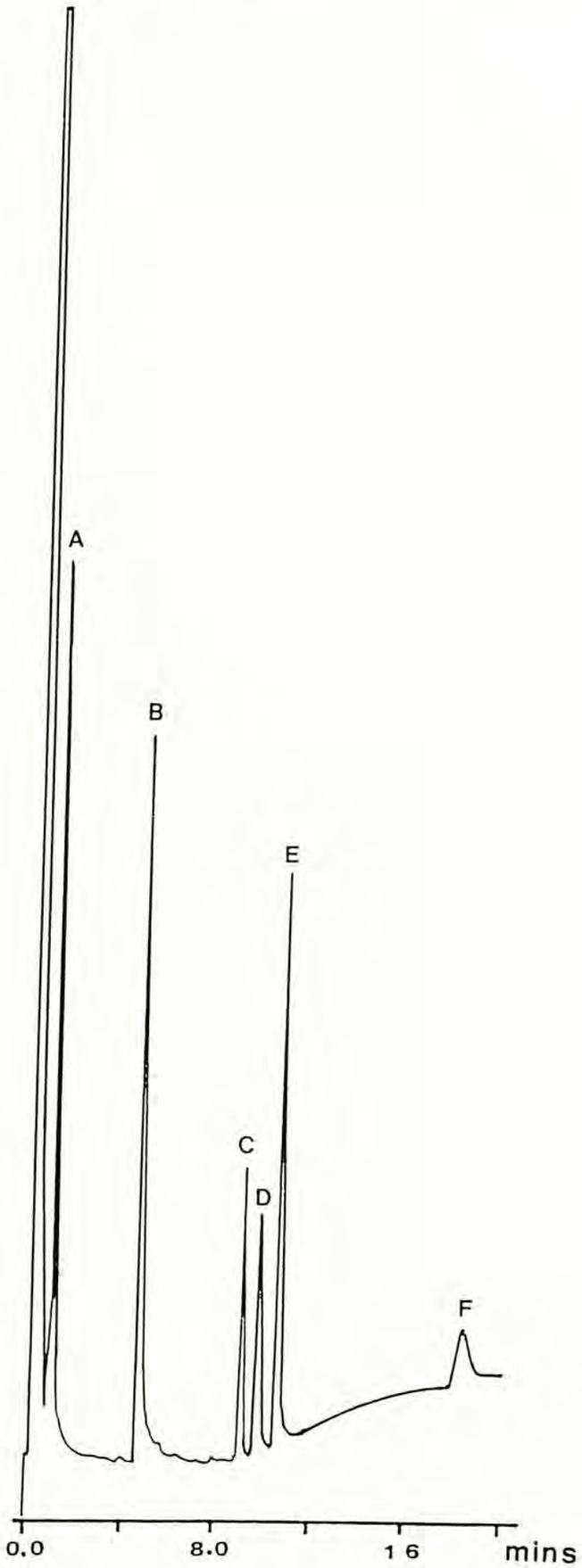
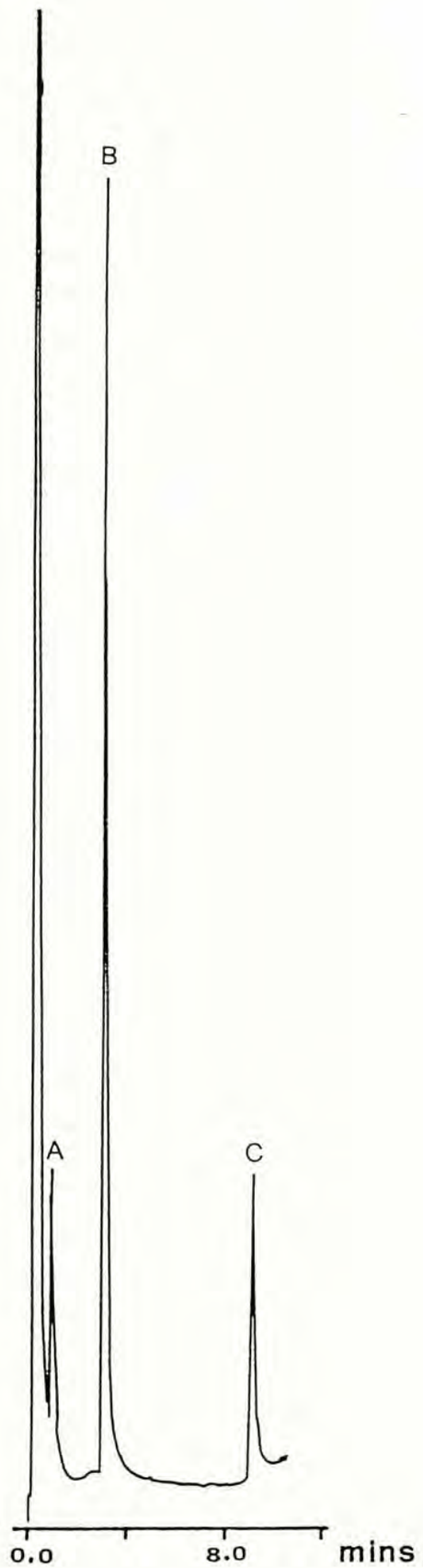


**Figure 2.3.6-2** The chromatogram of sample No.9, with peaks of A, pseudoephedrine hydrochloride; B, guaiphenesin; and C, clomipramine hydrochloride.



## 2.4 CONCLUSION

A simple, efficient, and accurate gas-liquid chromatographic method for the simultaneous determination of bromhexine, chlorpheniramine, codeine, dextromethorphan, diphenhydramine, ephedrine (or pseudoephedrine), guaiphenesin, and papaverine in cough-cold syrups has been developed. The proposed method involved a preliminary extraction with chloroform under alkaline conditions, which has been shown to be successful to extract the basic drugs from the sample matrices so that the interferences due to other excipients, such as dyes, flavours, and preservative present in samples, can be minimised. The time for running a chromatogram for the syrups was 10 min. or less when papaverine was absent, and about 20 min. when the latter was present. The method has been applied successfully and conveniently to the determination of the drugs mentioned above, individually or together, in nine commercial cough-cold syrups. The method is also suitable as a stability indicating assay.

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# CHAPTER 3

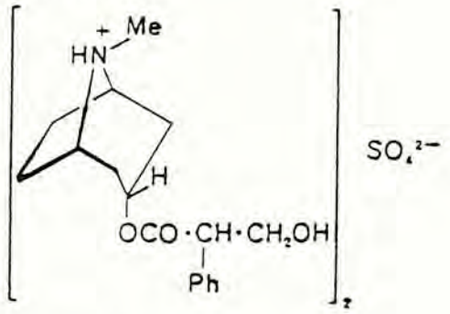
# GAS-LIQUID CHROMATOGRAPHIC DETERMINATION OF ATROPINE SULFATE/HYOSCYAMINE SULFATE, HOMATROPINE HYDROBROMIDE AND HYOSCINE HYDROBROMIDE IN PHARMACEUTICAL PREPARATIONS

## 3.1 INTRODUCTION

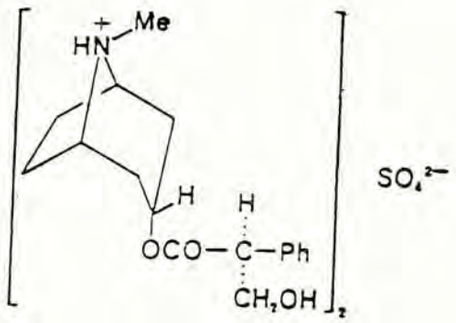
Atropine, homatropine, hyoscine (or scopolamine) and hyoscyamine are alkaloids have similar structures and properties. They are widely used in pharmaceutical preparations, such as eye drops, injections, and tablets, with quantities ranging from micrograms to milligrams. The actions and uses of these alkaloids are illustrated as follows[1-3]:

i. Atropine and hyoscyamine

Structure of atropine sulfate:



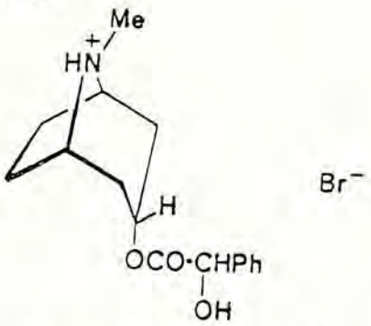

Structure of hyoscyamine sulfate:



Atropine is a mixture of (±)-hyoscyamine, prepared by racemisation of (-)-hyoscyamine, and thus, both atropine and hyoscyamine have the same properties. Atropine has central and peripheral actions. It first stimulates and then depresses the central nervous system and has antispasmodic actions on smooth muscle and reduces secretions, especially salivary and bronchial secretions. It also dilates the pupil, paralyses the muscles of accommodation, and increases intra-ocular pressure. In addition, as it diminishes gastric and intestinal motility, it is also used in the treatment of gastric and duodenal ulcer.

ii. Homatropine

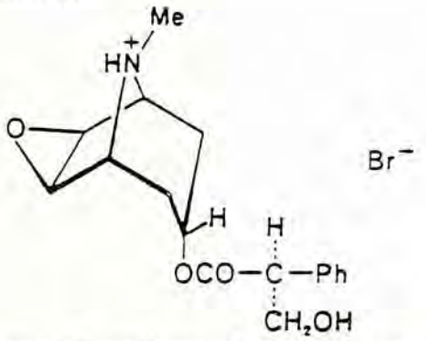
Structure of homatropine hydrobromide:




Homatropine has actions similar to those described under atropine. It is less potent than atropine and is rarely used internally, and its chief use is in ophthalmology to dilate the pupil. It produces mydriasis more rapidly than atropine, but its effect persists for a shorter time, and may be readily terminated by the action of physostigmine. It has less tendency than atropine to increase the intra-ocular pressure.

iii. Hyoscine (or Scopolamine)

Structure of hyoscine hydrobromide:




Hyoscine has peripheral and central actions. Same as atropine, it antagonises the muscarinic effects of acetylcholine. It produces mydriasis and relaxes accommodation more quickly but for a shorter time than atropine.

Numerous methods have been developed for the quantitative determination of these alkaloids mentioned, including derivative spectrophotometry[4], fluorometry[5,6], high performance liquid chromatography[5-8], gas-liquid chromatography[9-11], potentiometric titration[3] and spectrophotometry[12]. However, few of them could simultaneously determine all of the alkaloids under study. The spectrophotometric method required large sample size because of the low UV absorptivities of the alkaloids and also the low level of the alkaloids in pharmaceutical preparations. Fluorometric detectors were used in some HPLC methods[5,6] for trace analysis, however, many laboratories cannot afford the high costs of these equipments. The BP potentiometric titration method[3] lacks selectivity in the determination of the alkaloids. None of the GC methods are applicable for simultaneous analysis of all the alkaloids mentioned above. Although the USP method[11] used gas chromatography for the quantitative assay of the alkaloids, the method used homatropine HBr as the internal standard, and hence, its use was restricted to atropine, hyoscine and hyoscyamine only. Besides, this official method required a cumbersome preliminary extraction process.

The object of the present work was to develop a simple and efficient gas-liquid chromatographic method for the determination of the three* alkaloids, individually or together, in various pharmaceutical preparations. The extraction and chromatographic conditions for the analysis of these alkaloids under study were optimised. The proposed method had been applied to determine the contents of the alkaloids in seven commercially available pharmaceutical preparations. The results obtained by the proposed method were compared with those obtained using an established HPLC method[7] and the label claimed values.

*Since atropine is prepared by racemisation of (-)-hyoscyamine, both have the same properties and chromatographic behaviours, i.e. same peak shape and retention time. In case that both are present, the proposed method could only determine the total atropine-hyoscyamine contents.

## 3.2 EXPERIMENTAL

### 3.2.1 Instrumentation

#### 3.2.1.1 *Apparatus*

A Varian Model 3700 Gas Chromatograph equipped with a flame ionization detector, and attached to a Hitachi 833A Data Processor was used.

#### 3.2.1.2 *Gas chromatographic conditions*

A glass column (5ft. x 2 mm i.d.) was packed with 3% OV-225 on Supelcoport (80/100 mesh). The carrier gas was nitrogen with a flow rate of 30 ml/min. The flow rate of hydrogen and air were 30 and 300 ml/min., respectively. The injection port and detector temperatures were kept at 260 and 250 °C, respectively. The initial column temperature was kept at 220 °C for the first minute, then programmed to a final temperature of 260 °C at a heating rate of 15 °C/min., and then maintained at this temperature for 4 min. and 2 min., respectively, for samples with and without hyoscine. The attenuation was 1 in the gas chromatograph and varied in the data processor (8-10, depending on the concentration of the injected solution). The chart speed of the data processor was 2.5 mm/min.. When not in use, the column was kept at 50 °C with a nitrogen flow rate of 30 ml/min. While broadening of the peaks was observed, which was an indication of decomposition of the packing material, the first few inches of the packing material near the injection port was replaced, and the column could be used for about six months.

### 3.2.2 The Counter-check HPLC Method

The results obtained using the proposed method were compared with an established HPLC method[7]. In performing the counter-check method, the procedure of the original method was followed except that the reagent 20% tetramethylammonium hydroxide in methanol was replaced by 25% tetraethylammonium hydroxide in methanol since the former reagent was not available in our laboratory.

#### 3.2.2.1 *Instrumentation*

The liquid chromatograph consisted of a controller (Beckman, model 421A), a solvent pump (Beckman, model 110B), an injection system (Altex, 210 valve), an analytical column ALLTECH, Applied Science Labs, 5 $\mu$ Econosphere C18 Cartridge 250 x 4.6 mm, i.d., a detector (Beckman, model 163 variable wavelength) and an integrator (Beckman, model 427). The column was protected by a guard column packed with the same packing material.

Instrumental settings

| | |
|---|---|
| Flow rate | 1.0 ml/min |
| Wavelength | 220 nm |
| Chart speed | 0.5 cm/min. |
| Attenuation | 32 |

3.2.2.2 *Mobile phase*

A tetraethylammonium phosphate buffer (about 0.05M) was first prepared by mixing 23 ml of 25% tetraethylammonium hydroxide in methanol and 10 ml of 85% phosphoric acid in 500 ml of re-distilled water, adjusting to pH 2.0 with 25% tetraethylammonium hydroxide in methanol, and diluting to 1 litre with re-distilled water.

The mobile phase was prepared by mixing 525 ml of tetraethylammonium phosphate buffer with 250 ml of methanol.

3.2.3 Reagents

All drugs were of Pharmacopoeial or equivalent purity, and were used without further purification. All other reagents were of analytical grade.

3.2.3.1 *Internal standard solution*

An aqueous internal standard solution of diphenhydramine hydrochloride (214 μg/ml) was prepared by dissolving exactly 0.0107 g of the compound (Sigma Chemical Co.) in 50 ml of sulphuric acid (0.1M) in a calibrated flask.

3.2.3.2 *Stock solutions of the alkaloids*

Stock solution of atropine sulfate (200 μg/ml) was prepared by weighing exactly 0.01 g of atropine sulfate monohydrate (Janssen Chimica) in 50 ml of sulphuric acid (0.1M) in a calibrated flask.

Similarly, stock solutions of homatropine hydrobromide (200 μg/ml), hyoscine hydrobromide (200 μg/ml), and hyoscyamine sulfate (200 μg/ml) were prepared by dissolving appropriate amounts of the corresponding compounds (Sigma Chemical Co.) in 50 ml of sulphuric acid (0.1M) in V-flasks.

### 3.2.4 Standard Solutions of the Alkaloids

Standard solutions of each alkaloids (4-50 μg/ml) were prepared by appropriate dilution of the corresponding stock solution with sulphuric acid (0.1M) in 25-ml calibrated flasks containing exactly 1 ml of the internal standard solution.

### 3.2.5 Preparation of Sample Solution*

#### 3.2.5.1 *Atropine Sulfate or Hyoscyamine Sulfate Tablets*

Ten tablets of atropine sulfate (or two tablets of hyoscyamine sulfate) were weighed accurately and powdered finely. An amount equivalent to about 0.25 mg of atropine sulfate (or hyoscyamine sulfate) was weighed accurately and dissolved in about 20 ml of sulphuric acid (0.1M), and then shaken in an ultrasonic bath, with addition of ice to the bath, for about 15-30 min. The mixture was filtered and the filtrate was collected and transferred into a 25-ml V-flask containing 1 ml of the internal standard solution, and finally diluted to mark with sulphuric acid (0.1M).

3.2.5.2 *Atropine Sulfate Injections or Atropine Sulfate Eye Drops*

The Atropine Sulfate Eye Drop samples were first diluted with 0.1M sulphuric acid so that the concentrations of atropine sulfate were about 0.2 mg/ml (Solution I). An accurately measured volume of Solution I or the Atropine Sulfate Injection, equivalent to 1 mg of atropine sulfate, was transferred to a 25-ml calibrated flask, followed by the addition of 1 ml of the internal standard solution, and then diluted to the mark with sulphuric acid (0.1M).

3.2.5.3 *Homatropine Hydrobromide Eye Drops*

The Homatropine Hydrobromide Eye Drop samples were first diluted with 0.1M sulphuric acid so that the concentrations of homatropine hydrobromide were about 0.2 mg/ml (Solution I). An accurately measured volume of Solution I, equivalent to 0.8 mg of homatropine hydrobromide, was transferred to a 25-ml calibrated flask followed by the addition of 1 ml of the internal standard solution, and diluted to the mark with sulphuric acid (0.1M).

*No real samples with hyoscine hydrobromide was available for assay, however, a synthetic sample with hyoscine hydrobromide was determined by the proposed method and the result was shown in the section of Recovery Test.

3.2.6 Quantitative Determination of the Alkaloids Under Study in Various Pharmaceutical Preparations

An aliquot (3 ml) of the sample or standard solution was pipetted into a separating funnel followed by the addition of 1 ml of sodium hydroxide solution (1 M). The alkaloids in the alkaline aqueous solution was immediately extracted with 10 ml of chloroform and shaken for one minute. The organic layer was transferred, via a funnel containing 2-3 grams of anhydrous sodium sulphate supported by a filter paper (NO. 5A), into a 10-ml V-flask. The aqueous layer and the sodium sulphate were washed with small volumes of chloroform, and the washings were collected and transferred into the same V-flask. The solution in the V-flask was then evaporated to dryness under a stream of nitrogen in a water bath at a temperature of 30-35 °C. The residue was dissolved in 100 $\mu$l of chloroform, and kept cool by ice in order to avoid evaporation of solvent, and 2.0 $\mu$l of the resulting solution was injected onto the chromatograph with a 10-$\mu$l Hamilton syringe under the chromatographic conditions mentioned previously. The injection were performed in duplicate.

The calibration graph was obtained by plotting the peak area ratios (alkaloid to internal standard) against the concentrations of the alkaloid in the aqueous standard solutions.

The amount of each alkaloid in the sample was deduced from the respective calibration graph.

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Choice of Extraction Medium

Other than the alkaloids of interest, other excipients may be present in various pharmaceutical preparations, such as preservative(s) in eye drops and injections, stabilizer(s) in tablets, and/or other active ingredients. In order to develop a successful gas-liquid chromatographic method for the determination of the alkaloids under study, an efficient extraction method was essential to separate them from the excipients present in samples.

The alkaloids of interest were suggested to be extracted by organic solvent from alkaline media[13]. Hence, sodium hydroxide (1M) was used to provide an alkaline medium before extraction. It is important to extract the alkaloids immediately from the aqueous solution with the organic solvent after addition of the base because the alkaloids can easily be decomposed under a base-assisted esterification process. Several organic solvents, including chloroform, dichloromethane, diethylether, and hexane, were assessed for their suitability as the extraction medium.

An aqueous standard solution, which contained known amounts of atropine sulfate (7.98 μg/ml), homatropine hydrobromide (138.6 μg/ml), hyoscine hydrobromide (285.3 μg/ml), and the internal standard solution (30.96 μg/ml), was prepared. A 3-ml aliquot of the aqueous standard solution was made alkaline with sodium hydroxide (1M) and extracted with 20 ml of each solvent for 3 min. The organic layer was transferred to a V-flask via a funnel containing anhydrous sodium sulphate. The final solution, including the washings, was evaporated to dryness under nitrogen in a warm water bath (30-35 °C). All the residue was dissolved in 100 μl of chloroform, 2.0 μl of

which was injected onto the chromatograph under the chromatographic conditions described previously. The peak area ratios (alkaloid to internal standard) in various organic solvents are shown in Table 3.3.1-1. Because the peak area ratios so obtained were directly proportional to the amounts of alkaloids extracted from the aqueous to the organic phase, these ratios were used to compare the extraction capacity of each solvent.

From Table 3.3.1-1, it was found that the extraction capacities of chloroform and dichloromethane for each alkaloid were comparable, except for atropine sulfate, while hexane was the poorest extraction medium. It was expected that the extraordinary high signal of atropine obtained by extraction with dichloromethane would result in a much better sensitivity than that of chloroform. The calibration graphs of atropine sulfate obtained by extraction with chloroform and dichloromethane were then compared, which are shown in Figure 3.3.1-1. Figure 3.3.1-1 indicated that the slopes of both calibration graphs were similar, which meant that there was no significant difference in the sensitivities of atropine sulfate obtained by extraction with either solvent. Besides, the calibration graph obtained with dichloromethane as extraction medium exhibited a very large positive intercept, which was suspected to be the cause of the extraordinary high signal of atropine. Since the extraction capacities of chloroform and dichloromethane for each alkaloid were similar, and the former gave zero intercepts in calibration graphs for the alkaloids, chloroform was chosen to be the extraction medium for the subsequent determinations.

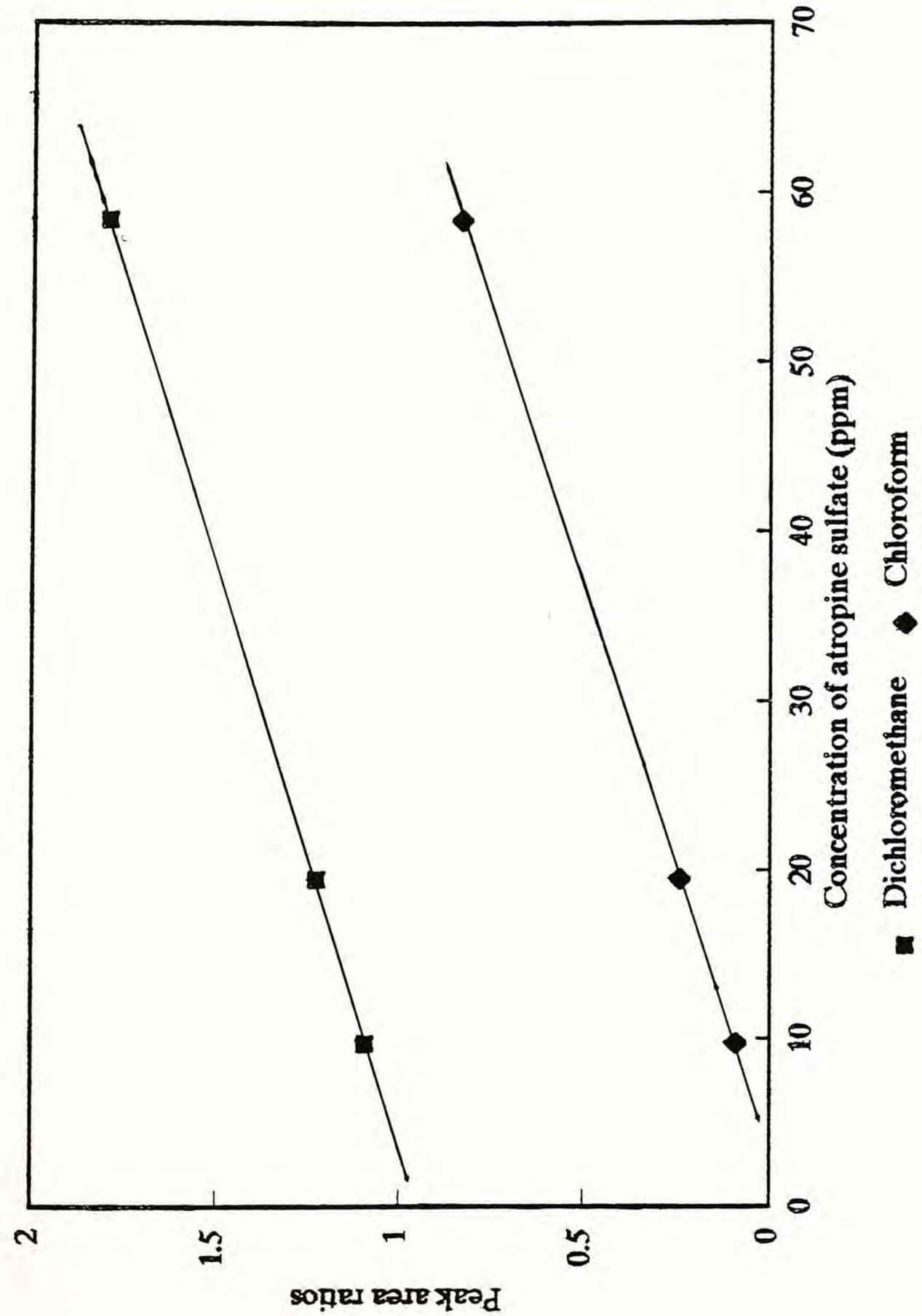
**Table 3.3.1-1** Peak area ratios (individual alkaloid to internal standard) after extraction with various solvents.

| Extraction medium | Peak area ratios of the alkaloids in the mixture* | | |
|---|---|---|---|
| | Atropine sulfate | Homatropine HBr | Hyoscine HBr |
| Chloroform | 0.1527 | 2.546 | 1.939 |
| Dichloromethane | 1.679 | 2.500 | 1.942 |
| Diethylether | 0.1135 | 1.803 | 1.496 |
| Hexane | 0.04526 | 1.037 | 0.07727 |

*The concentrations of the alkaloids in aqueous standard solution were as follows:

1. Atropine sulfate ( 7.98 μg/ml);
2. Homatropine hydrobromide (138.6 μg/ml);
3. Hyoscine hydrobromide (285.3 μg/ml).

**Figure 3.3.1-1** Comparison of the calibration graphs of atropine sulfate obtained by extraction with chloroform and dichloromethane, separately.



### 3.3.2 Optimisation of the volume of extraction medium and the shaking time

In order to save time and simplify the extraction procedure, a single extraction was proposed. Because the extraction was only performed once, the volume of extraction medium and the shaking time of the extraction became very critical.

An aqueous standard solution containing known amounts of atropine sulfate (11.66 μg/ml), homatropine HBr (23.76 μg/ml), hyoscine HBr (48.91 μg/ml), and internal standard was prepared. Aliquots (3 ml) of the aqueous standard solution were made alkaline and extracted separately with 10, 15 and 20 ml of chloroform at a fixed shaking time of 3 min. The organic layer was collected after being dried with anhydrous sodium sulphate, and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of chloroform, 2.0 μl of which was injected into the chromatograph. The peak area ratios (alkaloid to internal standard) obtained using various extraction volumes are shown in Table 3.3.2-1. From which, it can be seen that there was no significant difference in the peak area ratios as the volume of the extraction medium varied. For economic reasons, 10 ml of chloroform was used in the subsequent extraction procedure.

Similarly, the shaking time of the extraction was also optimised. The alkaloids in the aqueous standard solution was extracted with 10 ml of chloroform but using different shaking times, namely, 1, 3, and 5 minutes, the extraction procedure being the same as above. The effect of shaking time of extraction on the peak area ratios of the alkaloids is shown in Table 3.3.2-2. The results indicated that the peak area ratios was also not affected by the shaking time. In order to save time, the shaking time of 1 min. was chosen.

**Table 3.3.2-1** Effect of the volume of chloroform used for extraction on the peak area ratios (individual alkaloid to internal standard), the shaking time being 3 min.

| Compound* | Peak area ratios of the alkaloids in the mixture | | |
|---|---|---|---|
| | Extraction volume used, ml | | |
| | 10 | 15 | 20 |
| Atropine sulfate | 1.283 | 1.277 | 1.346 |
| Homatropine HBr | 2.380 | 2.369 | 2.359 |
| Hyoscine HBr | 3.874 | 3.892 | 3.839 |

*The concentrations of the alkaloids in aqueous standard solution were as follows:

1. Atropine sulfate (11.66 μg/ml);
2. Homatropine hydrobromide (23.76 μg/ml);
3. Hyoscine hydrobromide (48.91 μg/ml).

**Table 3.3.2-2** Effect of the shaking time on the peak area ratios (individual alkaloid to internal standard), the volume of chloroform being 10 ml.

| Compound* | Peak area ratios of the alkaloids in the mixture | | |
|---|---|---|---|
| | Shaking time used, min. | | |
| | 1 | 3 | 5 |
| Atropine sulfate | 2.452 | 2.414 | 2.399 |
| Homatropine HBr | 4.555 | 4.551 | 4.434 |
| Hyoscine HBr | 7.438 | 7.378 | 7.439 |

*The concentrations of the alkaloids in aqueous standard solution were as follows:

1. Atropine sulfate (19.44 μg/ml);
2. Homatropine hydrobromide (39.60 μg/ml);
3. Hyoscine hydrobromide (81.52 μg/ml).

### 3.3.3 Choice of stationary phase

As atropine is a mixture of (+)- and (-)-hyoscyamine, both compounds may be viewed to be the same in terms of chromatographic behaviour. Thus, only the alkaloids of atropine, homatropine and hyoscine needed to be resolved. In general, it is not difficult to find a suitable stationary phase for separating these three compounds, however, the problem became harder if the compounds are unstable to heat. It was earlier known that the alkaloids under study might be decomposed on the column[10]. The proper choice of stationary phase became very important because not only was it necessary to have good resolution for the alkaloids under study, but also the decomposition of the compounds needed to be minimized.

Several stationary phases on Supelcoport (80/100 mesh), including 3% OV-7, 3% OV-25, and 3% OV-225, were evaluated. The composition, McReynolds constants, and temperature limits of these stationary phases are shown in Table 3.3.3-1. Because of their high maximum temperature limits, they are suitable for separating these non-volatile alkaloids under study. The large difference in McReynolds Constants of various stationary phases indicated that their resolution power will not be the same. The retention times, peak shapes of the alkaloids under the specified chromatographic conditions for each column are listed in Table 3.3.3-2 to Table 3.3.3-4, and the respective chromatograms of the alkaloids are shown in Figure 3.3.3-1 to Figure 3.3.3-3.

**Table 3.3.3-1** The specifications of various stationary phases*.

| Phase | Composition | Temperature limit (°C) | McReynolds constants** | | | | |
|---|---|---|---|---|---|---|---|
| | | Min./Max. | X' | Y' | Z' | U' | S' |
| OV-7 | Phenyl methyl dimethyl, 20% phenyl | 0/350 | 69 | 113 | 111 | 171 | 128 |
| OV-25 | Phenyl methyl diphenyl, 75% phenyl | 0/350 | 178 | 204 | 208 | 305 | 280 |
| OV-225 | Cyanopropylmethyl -phenyl methyl | 0/265 | 228 | 369 | 338 | 492 | 386 |

*Reference : SUPELCO, International Catalog 25, 1987, pp.88.

**Test solutes :
X' = benzene;
Y' = 1-butanol;
Z' = methyl-n-propyl ketone;
U' = nitromethane; and
S' = pyridine.

**Table 3.3.3-2** The retention times and peak shapes of the alkaloids under study in column of **3% OV-7** under the specified conditions[+].

| Compounds | Retention times[*], min. | Relative peak area ratio | Remarks |
|---|---|---|---|
| Atropine sulfate/ hyoscyamine sulfate[**] | A: 3.17 (s)<br>B: 3.88 (s)<br>C: 5.26 (s) | A: 7.4<br>B: 5.5<br>C: 1.0 | Multi-peak, decomposition occurred. |
| Homatropine HBr | 4.18 (s) | nil | Single peak, no decomposition. |
| Hyoscine HBr | A: 4.27 (s)<br>B: 4.96 (s) | A: 1.5<br>B: 1.0 | Multi-peak, decomposition occurred. |

[+]Chromatographic conditions used :

| | | |
|---|---|---|
| Column used | : | 3% OV-7 (5ft. x 2 mm i.d.) on Supelcoport (80/100 mesh); |
| Initial temperature | : | 190 °C (1 min.); |
| Heating rate | : | 10 °C/min.; |
| Final temperature | : | 265 °C (1 min.). |

[*]Peak shape enclosed in parentheses: s, sharp.

[**]Atropine sulfate and hyoscyamine sulfate had the same retention time and peak shape.

**Figure 3.3.3-1**Gas chromatograms of the alkaloids obtained by using column of **3% OV-7** under the conditions mentioned in Table 3.3.3-2.

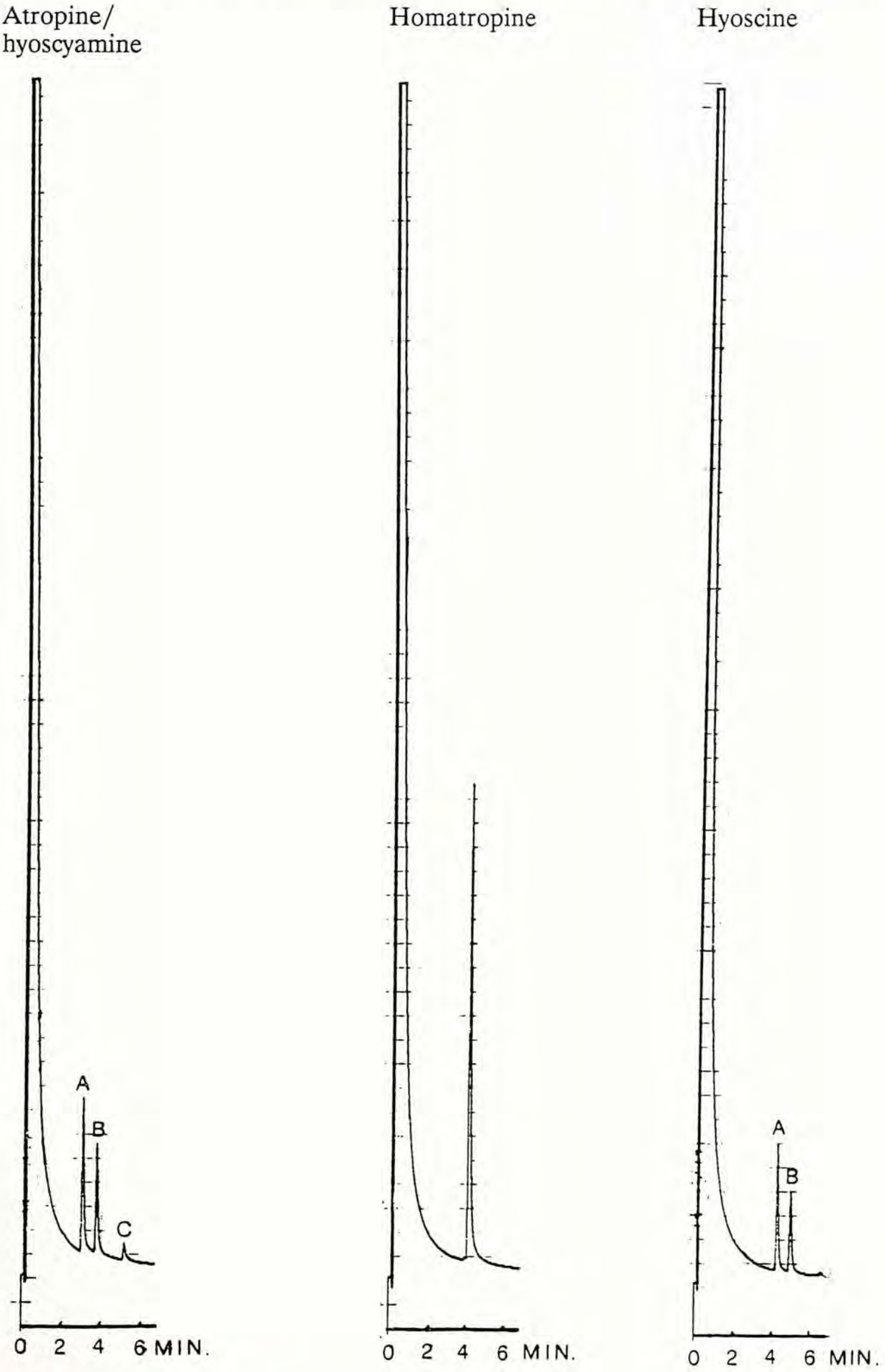


**Table 3.3.3-3** The retention times and peak shapes of the alkaloids under study in column of **3% OV-25** under the specified conditions[+].

| Compounds | Retention times[*], min. | Relative peak area ratio | Remarks |
|---|---|---|---|
| Atropine sulfate/ hyoscyamine sulfate[**] | A: 5.98 (s)<br>B: 6.89 (s)<br>C: 8.65 (s) | A: 16.0<br>B: 8.5<br>C: 1.0 | Multi-peak, decomposition occurred. |
| Homatropine HBr | 7.26 (s) | nil | Single peak, no decomposition. |
| Hyoscine HBr | A: 7.54 (s)<br>B: 8.36 (s) | A: 3.2<br>B: 1.0 | Multi-peak, decomposition occurred. |

[+]Chromatographic conditions used :

| | | |
|---|---|---|
| Column used | : | 3% OV-25 (5ft. x 2 mm i.d.) on Supelcoport (80/100 mesh); |
| Initial temperature | : | 170 °C (1 min.); |
| Heating rate | : | 10 °C/min.; |
| Final temperature | : | 265 °C (1 min.). |

[*]Peak shape enclosed in parentheses: s, sharp.

[**]Atropine sulfate and hyoscyamine sulfate had the same retention time and peak shape.

**Figure 3.3.3-2** Gas chromatograms of the alkaloids obtained by using column of **3% OV-25** under the conditions mentioned in Table 3.3.3-3.

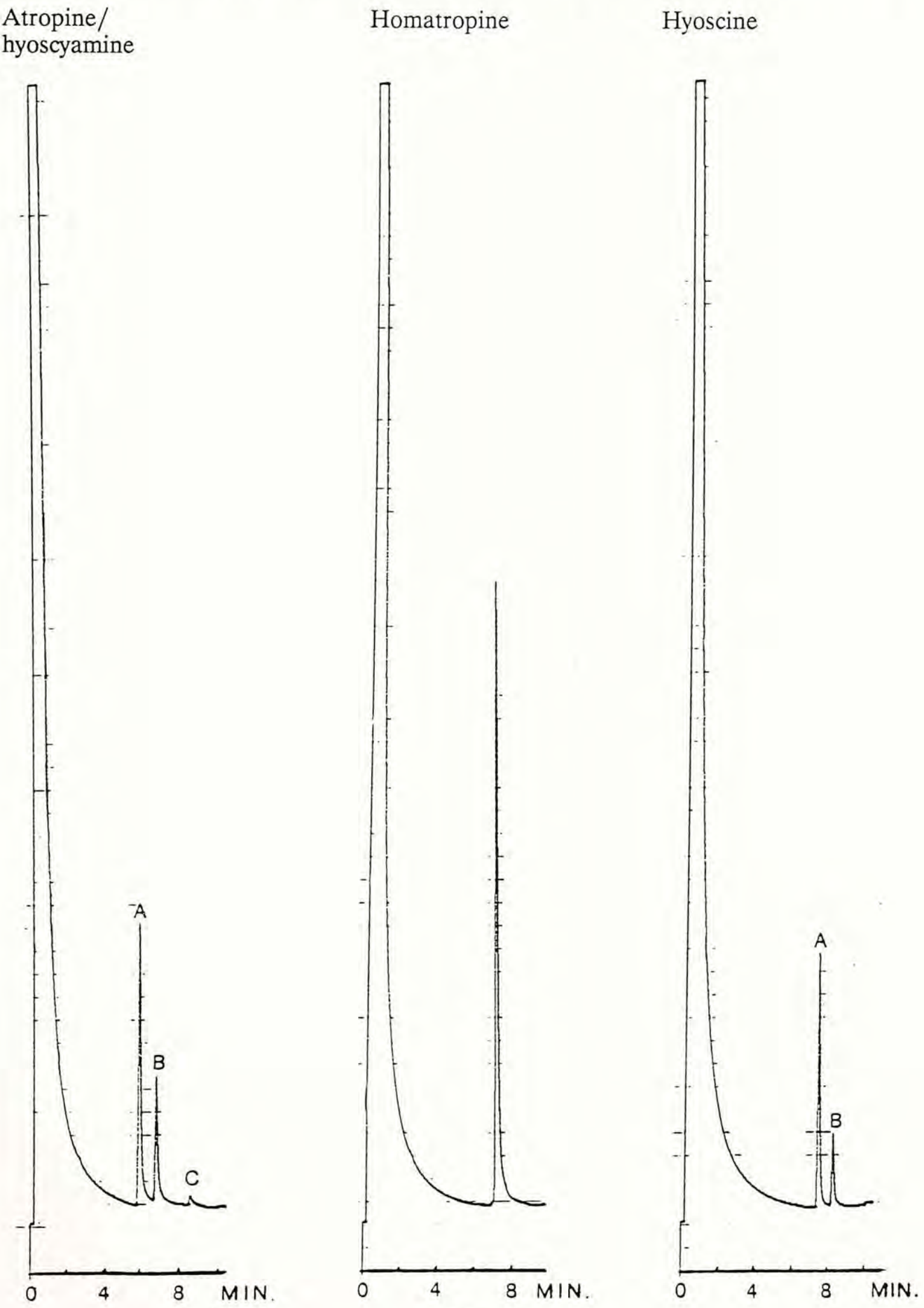


**Table 3.3.3-4** The retention times and peak shapes of the alkaloids under study in column of **3% OV-225** under the specified conditions[+].

| Compounds | Retention times[*], min. | Relative peak area ratio | Remarks |
|---|---|---|---|
| Atropine sulfate/ hyoscyamine sulfate[**] | A: 3.72 (s)<br>B: 4.32 (s)<br>C: 6.89 (s) | A: 1.1<br>B: 1.0<br>C: 8.8 | Multi-peak, decomposition occurred. |
| Homatropine HBr | 5.45 (s) | nil | Single peak, no decomposition. |
| Hyoscine HBr | A: 5.76 (s)<br>B: 6.34 (s)<br>C:10.37 (s) | A: 1.2<br>B: 1.0<br>C: 6.8 | Multi-peak, decomposition occurred. |

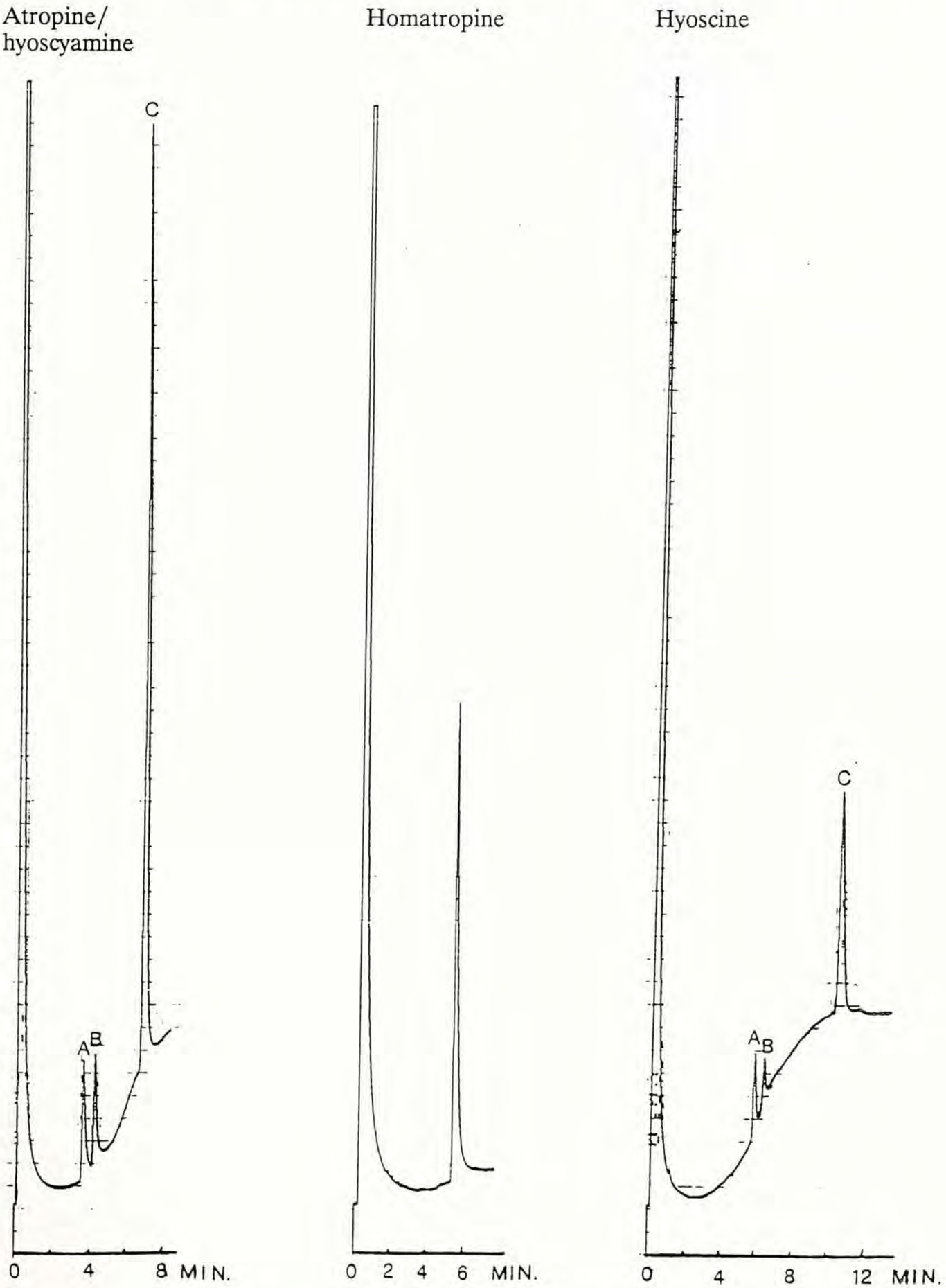
[+]Chromatographic conditions used :

| | | |
|---|---|---|
| Column used | : | 3% OV-225 (5ft. x 2 mm i.d.) on Supelcoport (80/100 mesh); |
| Initial temperature | : | 200 °C (1 min.); |
| Heating rate | : | 10 °C/min.; |
| Final temperature | : | 250 °C (1 min.). |

[*]Peak shape enclosed in parentheses: s, sharp.

[**]Atropine sulfate and hyoscyamine sulfate had the same retention time and peak shape.

**Figure 3.3.3-3**Gas chromatograms of the alkaloids obtained by using column of **3% OV-225** under the conditions mentioned in Table 3.3.3-4.



From Figure 3.3.3-1 to Figure 3.3.3-3, it was found that all the alkaloids under study, except homatropine, were most likely decomposed on the columns because of the observation of multi-peak for individual alkaloid. The decomposition products were expected to have a shorter retention time than that of the original compound. For example, Peak C shown on the chromatograms of atropine/hyoscyamine in various types of column might be the peak of the original compound while Peak A and Peak B were suspected due to the decomposition products. The relative peak area ratios (shown on Table 3.3.3-2 to Table 3.3.3-4) of these peaks illustrated the degree of decomposition of the compound in various types of column. Say, for atropine/hyoscyamine, the relative peak area ratios of Peak A and Peak B were greater than that of Peak C in the columns packed with 3% OV-7 or 3% OV-25, however, the relative peak area ratio of Peak C became dominant in the column of 3% OV-225. Similar observations were obtained for hyoscine obtained using various types of column. It is apparent that the degree of decomposition of the alkaloids on the column packed with 3% OV-225 was the least. As a result, the stationary phase of 3% OV-225 was chosen for further study.

Walters et al[10] reported that the decomposition of atropine could be avoided by using very little glass wool or none at all at the injection site end of the column. Thus, the glass wool at the injection site of the column packed with 3% OV-225 was removed. After removal of the glass wool, the retention times and peak shapes of the alkaloids under the optimised chromatographic conditions are listed on Table 3.3.3-5, and the typical chromatogram of the alkaloids were shown in Figure 3.3.3-4, where no small peaks was observed preceding the peaks of atropine, homatropine and hyoscine. Thus, the decomposition problem of the alkaloids in the column of 3% OV-225 seemed to be mainly due to the decomposition at the injection site and could be avoided by the removal of glass wool at the site. Finally, a column packed with 3% OV-225 and with

no glass wool at the injection site was used for subsequent determinations.

**Table 3.3.3-5** The retention times and peak shapes of the alkaloids under study in column of **3% OV-225 (without injection site glass wool)** under the optimised conditions[+].

| Compounds | Retention times[*], min. | Relative peak area ratio | Remarks |
|---|---|---|---|
| Homatropine HBr | 3.41 (s) | nil | Single peak, no decomposition. |
| Atropine sulfate/ hyoscyamine sulfate[**] | 4.35 (s) | nil | Single peak, no decomposition. |
| Hyoscine HBr | 6.71 (s) | nil | Single peak, no decomposition. |

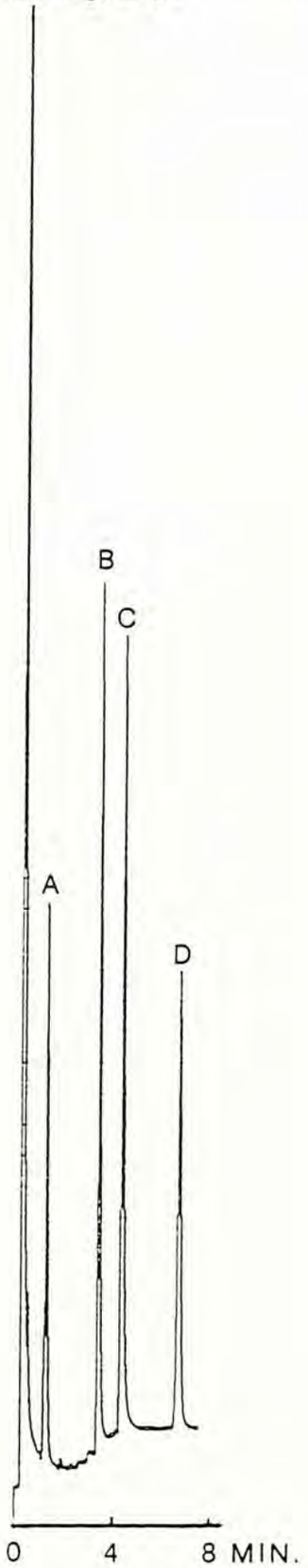
[+]Chromatographic conditions used :

| | | |
|---|---|---|
| Column used | : | 3% OV-225 (5ft. x 2 mm i.d.) on Supelcoport (80/100 mesh); |
| Initial temperature | : | 220 °C (1 min.); |
| Heating rate | : | 15 °C/min.; |
| Final temperature | : | 260 °C (4 min.). |

[*]Peak shape enclosed in parentheses: s, sharp.

[**]Atropine sulfate and hyoscyamine sulfate had the same retention time and peak shape.

**Figure 3.3.3-4** Typical chromatogram of the alkaloids obtained by using column of **3% OV-225 (without injection site glass wool)** under the optimised conditions with A, diphenhydramine HCl (internal standard, 8.56 μg/ml); B, homatropine HBr (34.50 μg/ml); C, atropine sulfate (31.94 μg/ml); and D, hyoscine HBr (34.56 μg/ml)*.




*Enclosed in parentheses was concentration before extraction.

### 3.3.4 Optimisation of The Instrumental Parameters

In gas chromatography, several instrumental parameters needed to be optimised: such as the flow rate of carrier gas, flow rates of hydrogen and air for the FID detector, and temperature programming conditions. Same as discussed in Chapter 2, the flow rate of carrier gas, nitrogen, was set to 30 ml/min, which is a common flow rate for analysis of drugs. In addition, the flow rates of hydrogen and air were optimised at 30 and 300 ml/min., respectively. After choosing the best stationary phase, the conditions of the temperature programming were optimised as followings.

An aqueous standard solution containing atropine sulfate (97.20 $\mu$g/ml), homatropine hydrobromide (198.0 $\mu$g/ml) and hyoscine hydrobromide (407.6 $\mu$g/ml) was prepared (Standard I). Standard I was diluted to 3.6, 5.0, 8.3, and 25 folds with sulphuric acid (0.1 M) in 25-ml V-flasks which contained 1 ml of the internal standard (Standards II). A series of standard solutions for injection were prepared from Standards II following the procedure described in the Experimental section. By fixing the initial and final temperature, the heating rate of the temperature programming was first optimised. For each heating rate, the extracted standard solutions were injected, and the calibration graph for each alkaloid was obtained. The effect of the heating rate on the slopes of the calibration graphs of the alkaloids are listed in Table 3.3.4-1. The change in the heating rate had no significant effect on the slopes of the calibration graphs (or sensitivities) of the alkaloids, however, the overall running time was slightly shorter at increasing heating rate. In order to obtain the shortest overall running time, a heating rate of 15 °C/min. was selected.

With fixed initial temperature and the optimised heating rate, the final temperature of the temperature programming was optimised. The effect of the final temperature on the slopes of the calibration graphs of the alkaloids is shown on Table

3.3.4-2. The slopes of the alkaloids were seen to gradually increase with increasing final temperature. Thus, a final temperature of 260 °C was chosen since it offered the best sensitivities for the alkaloids and the fastest overall running time.

After fixing the heating rate and final temperature, the optimisation of the initial temperature of the temperature programming was performed. The slopes of the calibration graphs of the alkaloids are shown on Table 3.3.4-3, where it can be seen that the slopes markedly increased with an increase in the initial temperature. Besides, the overall running time was also shortened when a higher initial temperature was employed. As a consequence, the initial temperature of 220 °C was chosen in order to gain a better response of the alkaloids and a shorter overall running time.

In order to evaluate the performance (or reproducibility) of the optimised temperature programming, a chloroform solution containing the alkaloids of atropine, homatropine, and hyoscine was prepared and injected ten times onto the column under the optimised chromatographic conditions, and the relative standard deviation of the peak area ratios for each alkaloid was then calculated. The results are shown in Table 3.3.4-4. The reproducibility of the optimised temperature programming can be considered as good because the relative standard deviation of each alkaloid ranged from 1.48 to 3.25%.

**Table 3.3.4-1** Effects of the heating rate of the temperature programming on the slopes of the calibration graphs of the alkaloids with fixed initial and final temperature*.

| Compounds | Slopes of the calibration graphs, ml/μg** | | |
|---|---|---|---|
| | Heating rate, °C/min | | |
| | 10 | 12 | 15 |
| Atropine sulfate | 0.09376 (5.60) | 0.09353 (5.30) | 0.09405 (5.00) |
| Homatropine HBr | 0.08498 (4.36) | 0.08513 (4.20) | 0.08576 (4.02) |
| Hyoscine HBr | 0.06950 (8.17) | 0.06967 (7.77) | 0.07016 (7.35) |

*The initial temperature was kept at 210 °C for 1 min.; and the final temperature was kept at 260 °C for 4 min.

**Enclosed in parentheses was the retention time in minute.

**Table 3.3.4-2** Effects of the final temperature of the temperature programming on the slopes of the calibration graphs of the alkaloids with fixed initial temperature and heating rate*.

| Compounds | Slopes of the calibration graphs, ml/μg** | | |
|---|---|---|---|
| | Final temperature, °C | | |
| | 250 | 255 | 260 |
| Atropine sulfate | 0.09105 (5.13) | 0.09299 (5.04) | 0.09405 (5.00) |
| Homatropine HBr | 0.08406 (4.04) | 0.08576 (4.02) | 0.08775 (4.02) |
| Hyoscine HBr | 0.06875 (8.25) | 0.06933 (7.74) | 0.07016 (7.35) |

*The initial temperature was kept at 210 °C for 1 min.; and the heating rate was kept at 15 °C/min.

**Enclosed in parentheses was the retention time in minute.

**Table 3.3.4-3** Effect of the initial temperature of the temperature programming on the slopes of the calibration graphs of the alkaloids with fixed final temperature and heating rate*.

| Compounds | Slopes of the calibration graphs, ml/$\mu$g** | | |
|---|---|---|---|
| | Initial temperature, °C | | |
| | 200 | 210 | 220 |
| Atropine sulfate | 0.09045<br>(5.70) | 0.09405<br>(5.00) | 0.1064<br>(4.30) |
| Homatropine HBr | 0.08058<br>(4.71) | 0.08576<br>(4.02) | 0.09837<br>(3.37) |
| Hyoscine HBr | 0.06510<br>(8.08) | 0.07016<br>(7.35) | 0.07791<br>(6.65) |

*The final temperature was kept at 260 °C for 4 min.; and the heating rate was kept at 15 °C/min.

**Enclosed in parentheses was the retention time in minute.

**Table 3.3.4-4** Data for testing the reproducibility of the optimised temperature programming.

| Compounds | Concentration in aqueous, $\mu$g/ml | Relative standard deviation* (%) |
|---|---|---|
| Atropine sulfate | 31.94 | 2.04 |
| Homatropine HBr | 34.50 | 1.83 |
| Hyoscine HBr | 34.56 | 1.48 |
| Hyoscyamine sulfate | 32.64 | 3.25 |

*For ten replicate injections.

3.3.5 Preparation of the calibration graphs

The calibration graph of each alkaloid under study was obtained by plotting the peak area ratios (alkaloid to internal standard) against the corresponding concentrations in aqueous standard solutions. In quantitative determination of the alkaloids, results were deduced from the calibration graphs with the help of the linear regression equations.

An aqueous standard solution, which contained atropine sulfate (199.2 $\mu$g/ml), homatropine hydrobromide (204.8 $\mu$g/ml) and hyoscine (199.2 $\mu$g/ml), was prepared by mixing appropriate amounts of the respective stock solutions in 50-ml calibrated flask (Standard I). Aliquots of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 13.0 ml of Standard I were diluted separately to 25-ml with sulphuric acid (0.1M) in calibrated flasks containing

1 ml of the internal standard solution (Standard II). Aliquot (3 ml) of Standard II was made alkaline with sodium hydroxide (1 M) and extracted with 10 ml of chloroform and hand shaken for 1 min. The organic layer was transferred, via a funnel containing anhydrous sodium sulphate, into a volumetric flask. The filtrate was evaporated to dryness under a stream of nitrogen in a warm water bath (30-35 °C). The residue was dissolved in 100 ul of chloroform, 2.0 $\mu$l of which was injected onto the chromatograph under the optimised conditions. The injection was performed in duplicate. The calibration graph for each alkaloid was obtained by plotting the peak area ratios (alkaloid to internal standard) against the corresponding concentrations in aqueous standard solution.

Similarly, the calibration graph of hyoscyamine sulfate was prepared with procedure described above.

The data for the calibration graphs of the alkaloids are listed in Table 3.3.5-1 to Table 3.3.5-4, and the respective calibration graphs are shown in Figure 3.3.5-1 to Figure 3.3.5-4.

**Table 3.3.5-1** Data on the calibration graph of atropine sulfate.

| Aqueous concentrations of atropine sulfate, μg/ml | Mean peak area ratios (n=2) |
|---|---|
| 3.98 | 0.5618 |
| 7.97 | 1.067 |
| 15.94 | 2.162 |
| 31.87 | 4.256 |
| 47.81 | 6.441 |
| 63.74 | 8.562 |
| 79.68 | 10.77 |
| 103.6 | 13.95 |

Linear working range = 4 - 104 μg/ml;

Slope = 0.1347 ml/μg;

Intercept = 0.001790;

Correlation coefficient = 0.9999;

Detection limit* = 4.0 μg/ml.

*The least detectable amount of alkaloid in aqueous samples.

**Figure 3.3.5-1** Calibration graph of atropine sulfate.

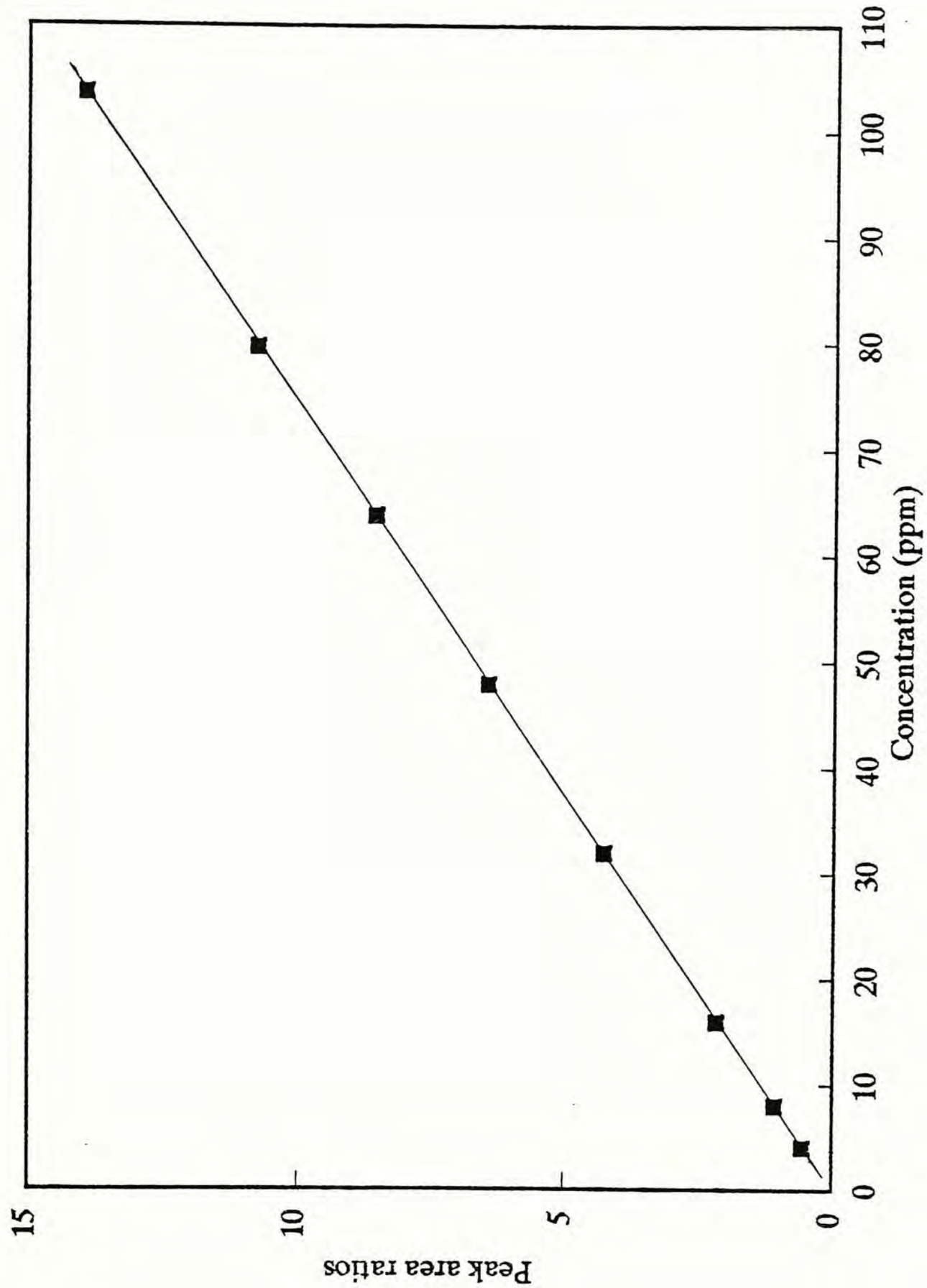


**Table 3.3.5-2** Data on the calibration graph of homatropine hydrobromide.

| Aqueous concentrations of homatropine HBr, μg/ml | Mean peak area ratios (n=2) |
|---|---|
| 4.10 | 0.4847 |
| 8.19 | 0.9425 |
| 16.38 | 1.936 |
| 32.77 | 3.903 |
| 49.15 | 6.000 |
| 65.54 | 7.994 |
| 81.92 | 10.04 |
| 106.5 | 13.06 |

Linear working range = 4 - 107 μg/ml;

Slope = 0.1232 ml/μg;

Intercept = -0.06916;

Correlation coefficient = 0.9999;

Detection limit = 4.0 μg/ml.

**Figure 3.3.5-2** Calibration graph of homatropine hydrobromide.

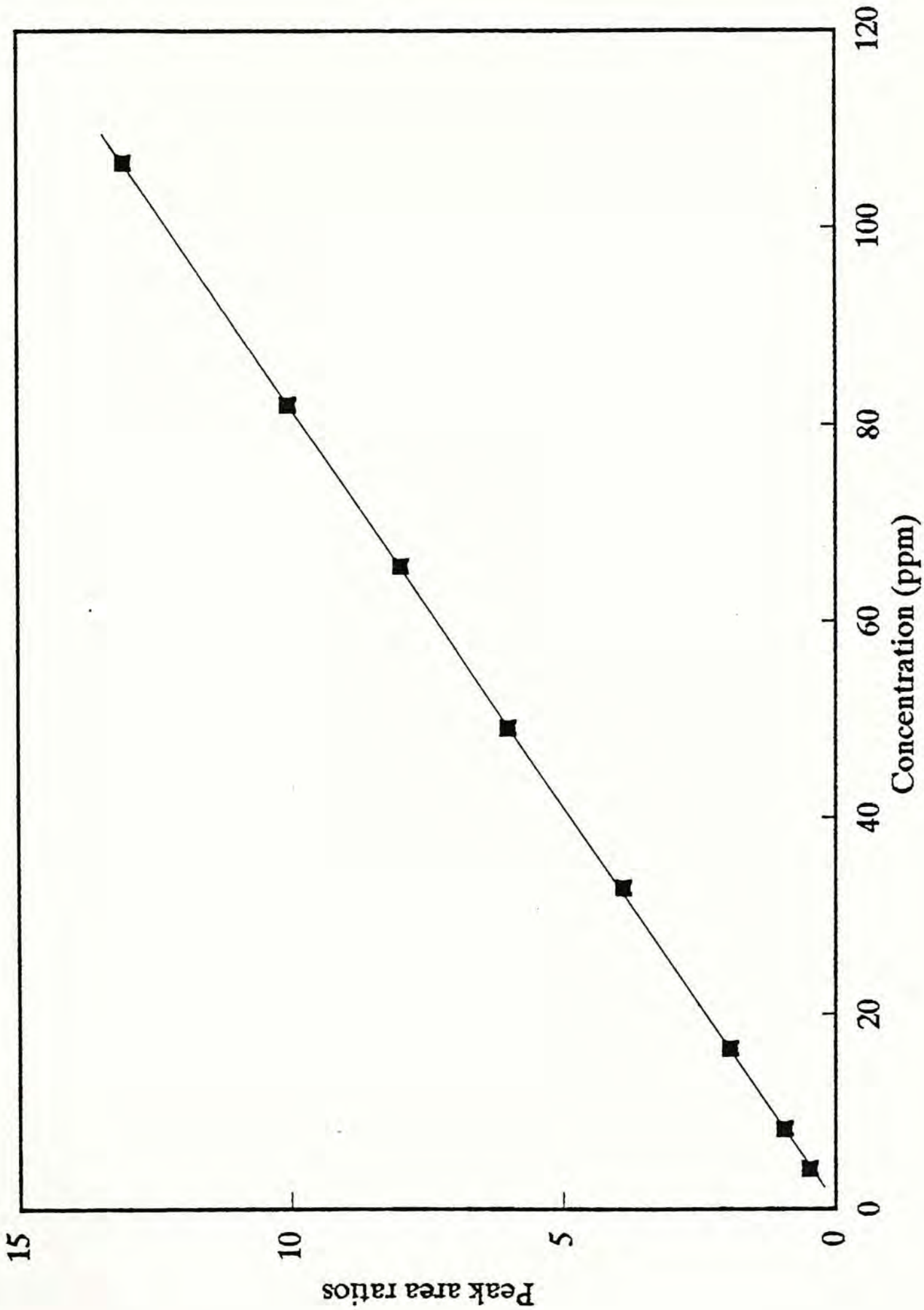


**Table 3.3.5-3** Data for the calibration graph of hyoscine hydrobromide.

| Aqueous concentrations of hyoscine HBr, μg/ml | Mean peak area ratios (n=2) |
|---|---|
| 3.98 | 0.3411 |
| 7.97 | 0.6670 |
| 15.94 | 1.419 |
| 31.87 | 2.863 |
| 47.81 | 4.571 |
| 63.74 | 6.085 |
| 79.68 | 7.713 |
| 103.6 | 9.959 |

Linear working range = 4 - 104 μg/ml;

Slope = 0.09745 ml/μg;

Intercept = -0.1171;

Correlation coefficient = 0.9998;

Detection limit = 4.0 μg/ml.

**Figure 3.3.5-3** Calibration graph of hyoscine hydrobromide.

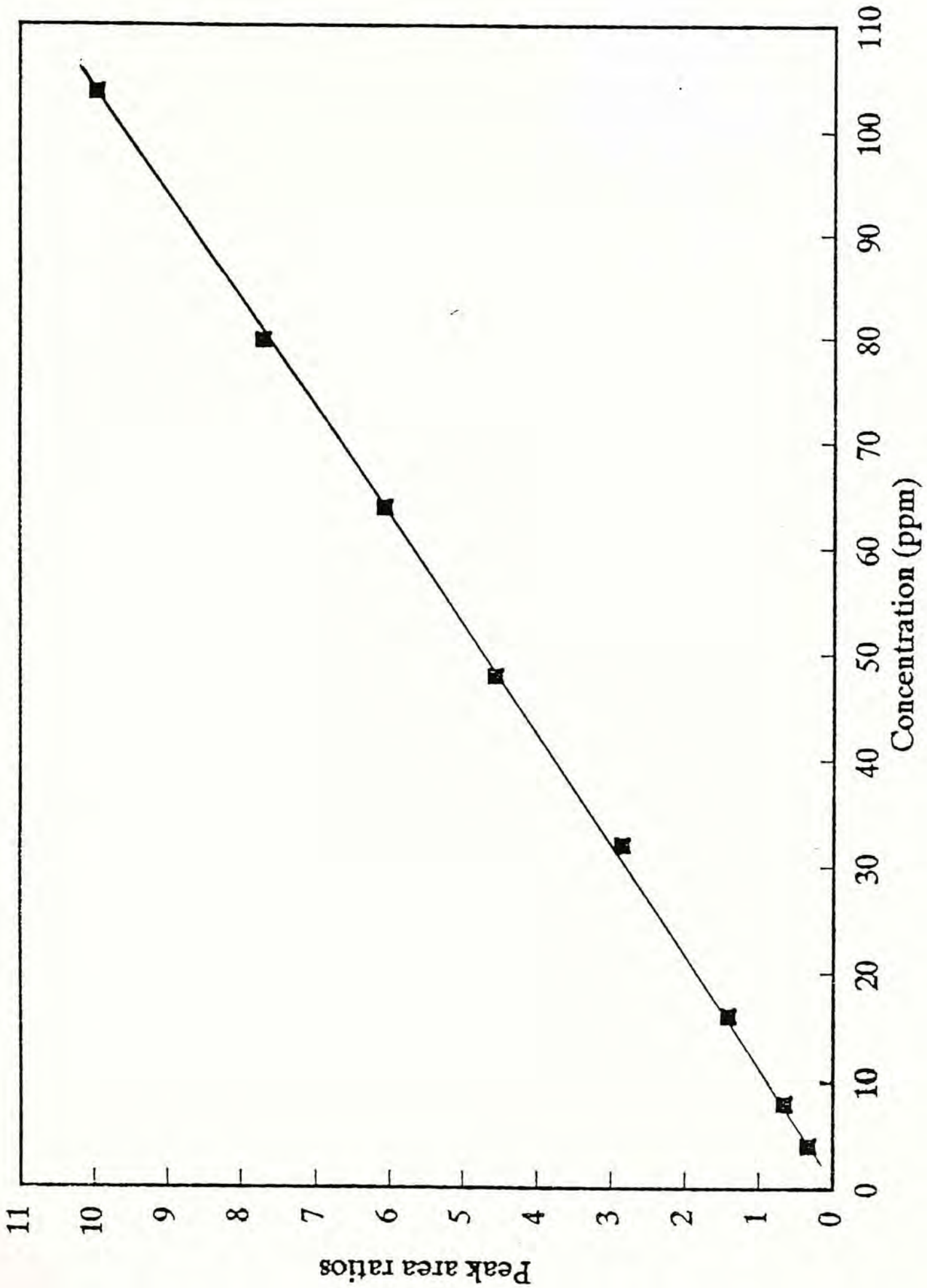


**Table 3.3.5-4** Data for the calibration graph of hyoscyamine sulfate.

| Aqueous concentrations of hyoscyamine sulfate, μg/ml | Mean peak area ratios (n=2) |
|---|---|
| 4.30 | 0.5046 |
| 8.61 | 1.307 |
| 17.22 | 2.450 |
| 34.43 | 5.118 |
| 51.65 | 7.605 |
| 68.86 | 10.15 |
| 86.08 | 12.85 |
| 111.9 | 16.68 |

Linear working range = 4 - 112 μg/ml;

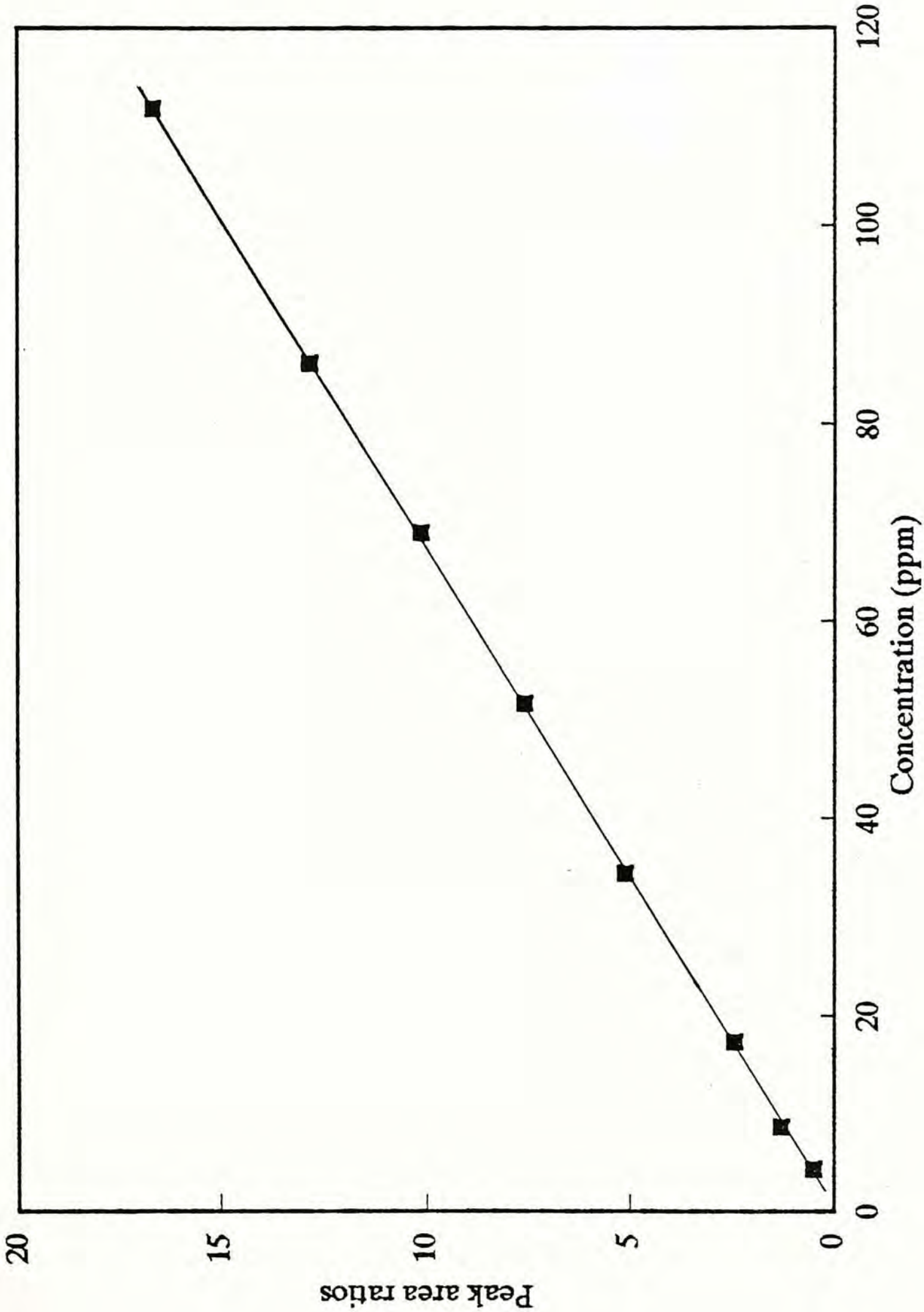
Slope = 0.1497 ml/μg;

Intercept = -0.08476;

Correlation coefficient = 0.9999;

Detection limit = 4.0 μg/ml.

**Figure 3.3.5-4** Calibration graph of hyoscyamine sulfate.



### 3.3.6 Recovery Test and Precision

The reliability of the proposed method was checked by performing the recovery tests on the alkaloids under study. The recovery tests were done by adding known amount of each alkaloid to tablet where it was known to be absent. For each alkaloid, four replicate determinations were performed, and the detailed procedure was described below.

An aqueous standard solution containing known amounts of atropine sulfate (199.6 μg/ml), homatropine hydrobromide (215.6 μg/ml) and hyoscine hydrobromide (216.0 μg/ml) was prepared (Standard I). A series of aqueous standard solutions containing the internal standard was prepared from Standard I (Standard II). A 3-ml aliquot of Standard I was pipetted into a beaker containing about 0.5 g of finely powdered tablet where the alkaloids were absent, and the mixture was made up to about 20 ml with sulphuric acid (0.1M) and shaken in an ultrasonic bath for 15-30 min. The mixture was filtered, and the filtrate was diluted to 25 ml with sulphuric acid (0.1M) in a calibrated flask containing 1 ml of the internal standard solution (Sample I).

A 3-ml aliquot of Sample I or Standard II was made alkaline with sodium hydroxide (1M) and extracted with 10 ml of chloroform and hand shaken for 1 min. The organic layer was transferred, via a funnel containing anhydrous sodium sulphate, into a volumetric flask. The filtrate was evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of chloroform, and 2.0 μl of which was injected on to the chromatograph under the optimised conditions. The amount of each alkaloid in Sample I was deduced from the respective calibration graph. The recovery test of hyoscyamine sulfate was performed in a similar way. The percentage recoveries are listed in Table 3.3.6-1. The mean results of four analyses ranged from 95.93 to 102.1%.

The precision of the proposed method was checked by calculating the relative standard deviation for the above determinations, and the results are also present in Table 3.3.6-1. The relative standard deviation of four analyses for each alkaloid under study ranged from 1.1 to 2.5%.

**Table 3.3.6-1** Results of the recovery tests and precision for the alkaloids under study using the proposed method.

| Drug | Amount added (mg/5 ml) | Recovery* (%) | Mean recovery (%) | Relative standard deviation (%) |
|---|---|---|---|---|
| Atropine sulfate | 23.95 | 102.0<br>102.8<br>103.1<br>100.3 | 102.1 | 1.1 |
| Homatropine HBr | 25.87 | 98.57<br>100.2<br>98.88<br>96.29 | 98.49 | 1.4 |
| Hyoscine HBr | 25.92 | 99.69<br>100.8<br>102.4<br>103.1 | 101.5 | 1.3 |
| Hyoscyamine sulfate | 24.48 | 94.82<br>94.91<br>93.87<br>100.1 | 95.93 | 2.5 |

### 3.3.7 Determination of The Alkaloids in Various Pharmaceutical Preparations

The contents of the alkaloids under study in seven commercially available pharmaceutical preparations were analysed in triplicate using the proposed method. The results obtained were compared with those obtained using an established HPLC method[7] and with the corresponding label values. A brief description of each of the sample was shown in Table 3.3.7-1. The assay results are listed in Table 3.3.7-2. Typical chromatograms for sample No.3, 6 and 7 are shown in Figure 3.3.7-1 to Figure 3.3.7-3, where no extra peaks due to other excipients were detected.

The average assay results of the proposed method were generally agreed with those obtained using the counter-check HPLC method and with the corresponding label values.

**Table 3.3.7-1** Brief description of the samples.

| Batch No. | Name* | Source | Ingredients | Labelled amount (mg/tablet) |
|---|---|---|---|---|
| 1 | ATROPINE (Eye drop) | Commercially available | Atropine sulfate<br>Benzalkonium chloride<br>Hydroxypropyl-methylcellulosum | 1 %<br>0.01 % |
| 2 | LOMOTIL (Tablet) | Private doctor | Atropine sulfate<br>Diphenoxylate HCl | 0.025<br>2.5 |
| 3 | LOMOTIL (Tablet) | Commercially available | Atropine sulfate<br>Diphenoxylate HCl | 0.025<br>2.5 |
| 4 | LOMOFEN (Tablet) | Commercially available | Atropine sulfate<br>Diphenoxylate HCl<br>Furazolidone | 0.025<br>2.5<br>50.0 |
| 5 | (Injection) | University Health Centre CUHK | Atropine sulfate | 0.6 mg/ml |
| 6 | (Eye drop) | University Health Centre CUHK | Homatropine HBr | 2 %<br>or 20 mg/ml |
| 7 | LEVSIN (Tablet) | Commercially available | (-)-Hyoscyamine sulfate | 0.125 |

*The form of the sample was enclosed in the parentheses.

**Table 3.3.7-2** The assay results for the determination of the alkaloids under study in various pharmaceutical preparations.

| Batch No. | Ingredients | Labelled values (mg/tablet) | Percentage of label claim | | |
|---|---|---|---|---|---|
| | | | By proposed method | | By HPLC method |
| | | | amount found | mean* | |
| 1 | Atropine sulfate | 1 % | 89.0<br>89.6<br>90.3 | 89.6 (0.5) | 92.9 |
| 2 | Atropine sulfate | 0.025 | 99.2<br>96.9<br>97.6 | 97.9 (1.0) | 94.4 |
| 3 | Atropine sulfate | 0.025 | 97.6<br>104.8<br>97.6 | 100.0 (3.4) | 100.8 |
| 4 | Atropine sulfate | 0.025 | 99.4<br>99.5<br>97.7 | 98.9 (0.83) | 95.6 |
| 5 | Atropine sulfate | 0.6 mg/ml | 114.5<br>113.6<br>111.5 | 113.2 (1.3) | 111.0 |
| 6 | Homatropine HBr | 2 % | 88.5<br>90.0<br>87.5 | 88.7 (1.0) | 90.5 |
| 7 | Hyoscyamine sulfate | 0.125 | 110.1<br>110.8<br>105.2 | 108.7 (2.5) | 108.8 |

**Figure 3.3.7-1** The chromatogram of sample No.3, with peaks of A, diphenhydramine hydrochloride; and B, atropine sulfate.

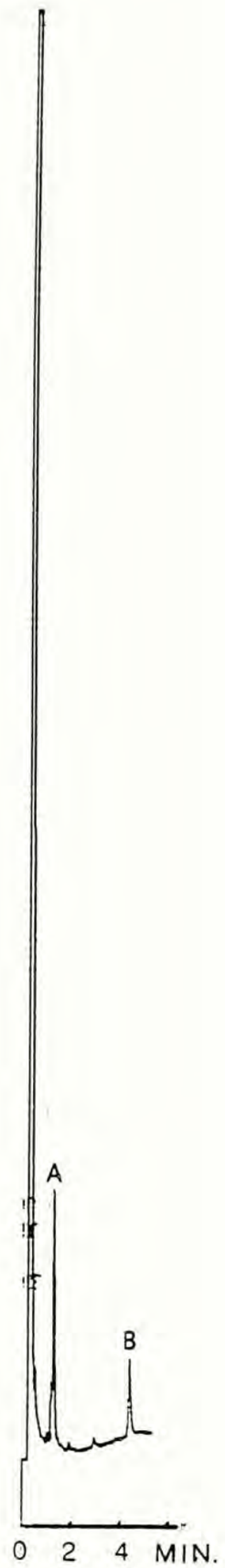


**Figure 3.3.7-2** The chromatogram of sample No.6, with peaks of A, diphenhydramine hydrochloride; and B, homatropine hydrobromide.

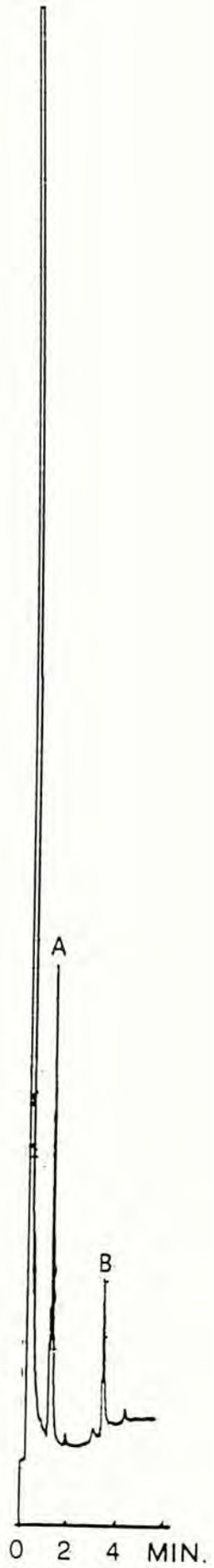


**Figure 3.3.7-3** The chromatogram of sample No.7, with peaks of A, diphenhydramine hydrochloride; and B, hyoscyamine sulfate.

## 3.4 CONCLUSION

A simple, efficient, and accurate gas-liquid chromatographic method for the simultaneous determination of atropine (or hyoscyamine), homatropine and hyoscine in pharmaceutical preparations has been developed. The proposed method used chloroform as the extraction medium, which could extract the alkaloids from various pharmaceutical preparations, such as eye drops, injections, and tablets, but not the excipients. The proposed extraction method offered the advantages of being fast and simple since a single-step extraction with chloroform was used with a shaking time of one minute. Besides, the detection limit of the proposed method was down to 4 $\mu$g/ml for all alkaloids under study, and thus the proposed method is suited for the assay of pharmaceutical preparations containing low levels of the alkaloids. The method has been applied successfully and conveniently to the determination of the alkaloids described above in seven pharmaceutical preparations, including eye drops, injection, and tablets.

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# APPENDIX

# MODIFICATION OF AN HPLC METHOD FOR DETERMINATION OF GUAIPHENESIN IN COUGH-COLD SYRUPS

## A.1 INTRODUCTION

Guaiphenesin, a common drug found in cough syrup formulations, is used to reduce the viscosity of tenacious sputum. Many methods have been established for the determination of guaiphenesin in pharmaceutical formulations, such as high performance liquid chromatographic (HPLC) and spectrophotometric methods.

Recently, Lau et al developed a reversed-phase ion-pair HPLC method[1] for the determination of the active ingredients present in cough-cold syrups, however, the method was not found to be suitable for the assay of guaiphenesin because it has a very short retention time and its peak is masked by the peaks of the dyes present in the syrups.

In order to overcome the masking effect due to the dyes, the simplest way was to remove the dyes from the sample matrix by solvent extraction, however, such preliminary procedure will complicate the original simple and convenient method. Another alternative was to minimize the effect of the dyes by dilution, and it was found that the sample solution was nearly colorless and the peak due to the dye(s) was hardly seen as the sample was diluted to 300-1000 folds. At such high dilution, the concentration of guaiphenesin in the sample solutions was around 20-30 ppm.

It was the purpose of the present work to modify the method of Lau et al[1] so that guaiphenesin in cough-cold syrups can be determined accurately. We have found that if the wavelength for the absorbance measurements is set at 274 nm, and the water content of the mobile phase is also increased to increase the retention time of guaiphenesin to achieve better resolution, it is possible to determine guaiphenesin with concentrations around 20 ppm.

The modified method was used as a counter-check method for the assay of guaiphenesin by the proposed GC method. The method was also successfully applied to analyse the content of guaiphenesin in five commercially available cough-cold syrups, and the results were shown in good agreement with the one obtained using the proposed GC method and also the label claimed values.

## A.2 EXPERIMENTAL

### A.2.1 Instumentation

The liquid chromatograph consisted of a controller (Beckman, model 421A), a solvent pump (Beckman, model 110B), an injection system (Altex, 210 valve), an analytical column Beckman 5 $\mu$ Ultrasphere-ODS 250 x 4.6 mm, i.d., a detector (Beckman, model 163 variable wavelength) and an integrator (Beckman, model 427). The column was protected by a guard column packed with the same packing material.

Instrumental settings

| | | |
|---|---|---|
| Flow rate | : | 1.0 ml/min. |
| Wavelength | : | 274 nm |
| Chart speed | : | 0.5 cm/min. |
| Attenuation | : | 16 |

A.2.2 Mobile phase

Composition of the mobile phase :

| | Original | New |
|---|---|---|
| Methanol | 715 ml | 635 ml |
| Water | 234 ml | 315 ml |
| Tetrahydrofuran | 50 ml | 50 ml |
| Phosphoric acid(85%) | 1 ml | 1 ml |
| Sodium dioctyl-sulphosuccinate | 5.8 g | 5.8 g |
| pH (adjusted by ammonia) | 4.6 | 4.6 |

The mobile phase was filtered through a 0.45 $\mu$m filter (Millipore). Finally, the mobile phase was degassed by suction and sonication, and then it was stored in an air-tight bottle.

A.2.3 Standard solution

Stock solution of guaiphenesin (504 ppm) was prepared by dissolving accurately 0.0504 g of the compound in 100 ml of the mobile phase in a volumetric flask. The standard solutions (5-30 ppm) were prepared by appropriate dilution of the stock solution with the mobile phase.

A.2.4 Sample solution

Sample solution I with about 500 - 1000 ppm of guaiphenesin was prepared by diluting appropriate amount of sample into 10 ml of the mobile phase in a calibrated flask. Sample solution II (used for injection) containing about 15 - 20 ppm of guaiphenesin was prepared by diluting appropriate amount of sample solution I into 10 ml of the mobile phase in a calibrated flask.

All the standard and sample solutions were filtered with a syringe equipped with a piece of 0.45 $\mu$m filter paper before injection.

A.2.5 Assay

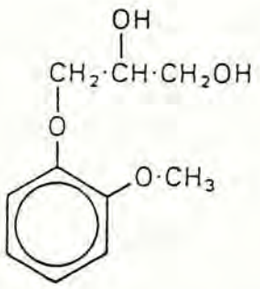
A sample/standard solution (20 $\mu$l) was injected into the system and the signals were recorded as peaks and their peak heights recorded by the integrator.

The calibration graph was plotted with the peak heights of guaiphenesin against its corresponding concentrations. The amount of guaiphenesin in each sample was deduced by interpolation on the calibration graph.

## A.3 RESULTS AND DISCUSSION

### A.3.1 Composition of the mobile phase

Considering the structure of guaiphenesin:




it can be seen that guaiphenesin lacks functional groups for protonation so that it cannot interact strongly with the ion-pair agent, and its retention time is rather short because of its weak interaction with the column material. In order to improve the peak resolution between guaiphenesin and other excipients such as dyes, the water content of the mobile phase was increased from 23.5 %, v/v to 31.5 %, v/v. Higher percentage of water content in the mobile phase can result in increasing the back pressure of the column, and thus, the flow rate of the mobile phase was reduced from 1.3 ml/min to 1.0 ml/min. in order to compensate for the effect of the increasing back pressure.

A.3.2 Effect of dilution

The dilution factor of the samples was optimised in order to successfully reduce the interference of the dyes. A real sample was selected and successively diluted to 10-fold, 20-fold, 33-fold, 100-fold and 333-fold, respectively, with the mobile phase. The chromatograms of the diluted samples were obtained using the modified method and are shown in Figure A.3.1-1 to Figure A.3.1-5. As the dilution factor of the sample was increased, the peak height of Peak B (suspected to be due to the dyes) gradually decreased and Peak B finally disappeared at 333-fold dilution. This observation suggested that the interference of dyes could be removed at a dilution of 333-fold or higher. In fact, the sample solution was seen to be colorless as the sample was diluted to 333 - 1000 folds.

**Figure A.3.1-1** The HPLC chromatogram of a 10-fold diluted sample No.1 with peaks of excipients (A, B, D, E, and F) and peak of guaiphenesin (C).

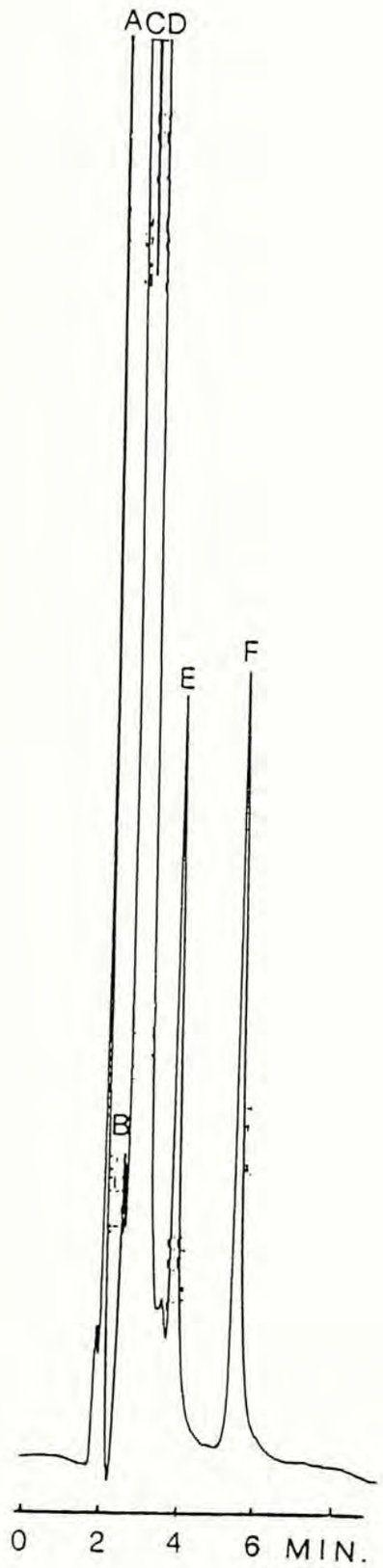


**Figure A.3.1-2** The HPLC chromatogram of a 20-fold diluted sample No.1 with peaks of excipients (A, B, D, E, and F) and peak of guaiphenesin (C).

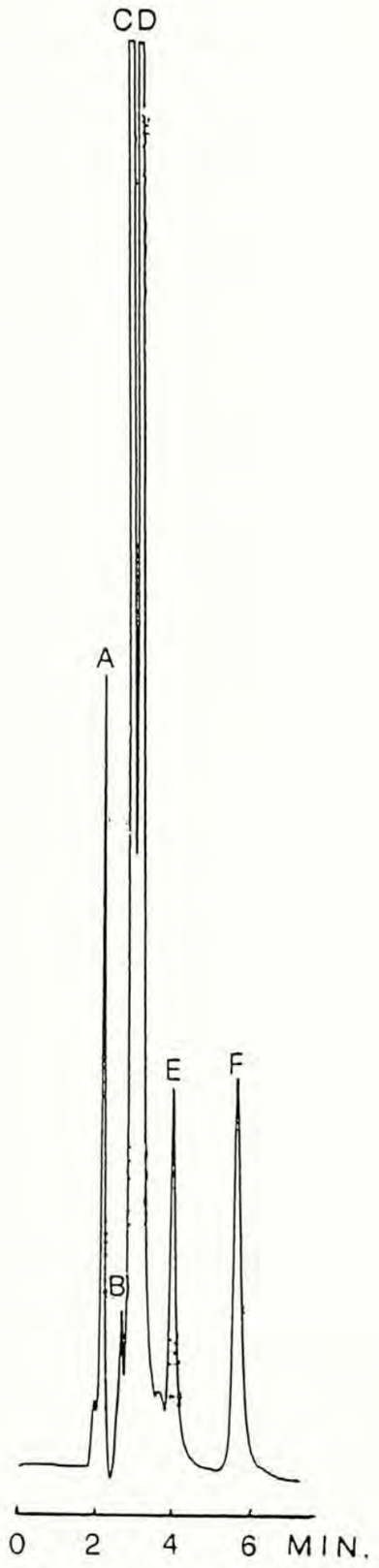


**Figure A.3.1-3** The HPLC chromatogram of a 33-fold diluted sample No.1 with peaks of excipients (A, B, D, E, and F) and peak of guaiphenesin (C).

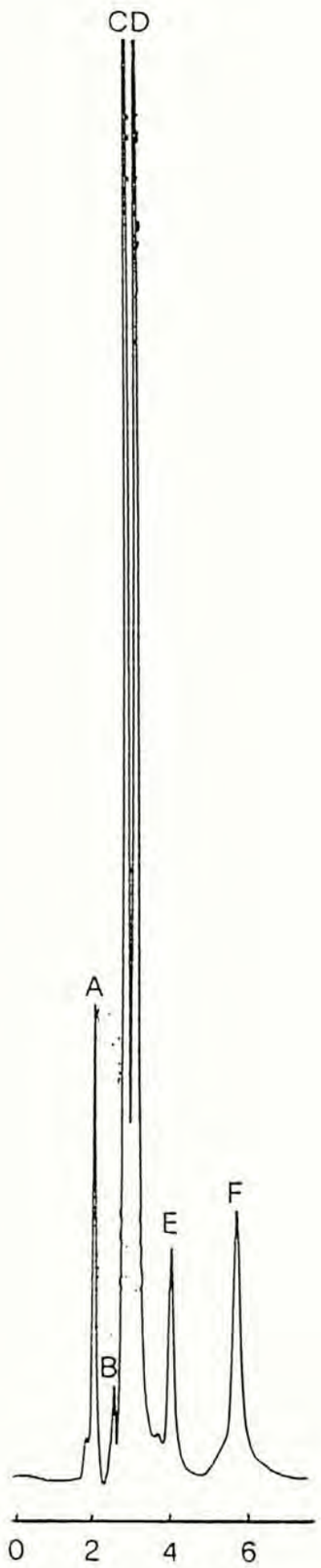


**Figure A.3.1-4** The HPLC chromatogram of a 100-fold diluted sample No.1 with peaks of excipients (A, B, D, E, and F) and peak of guaiphenesin (C).

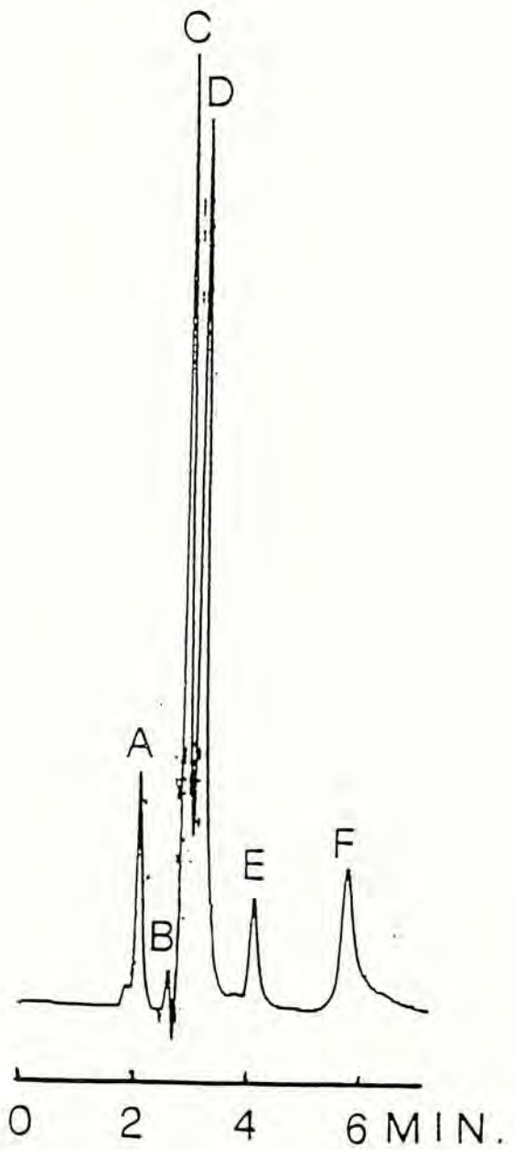
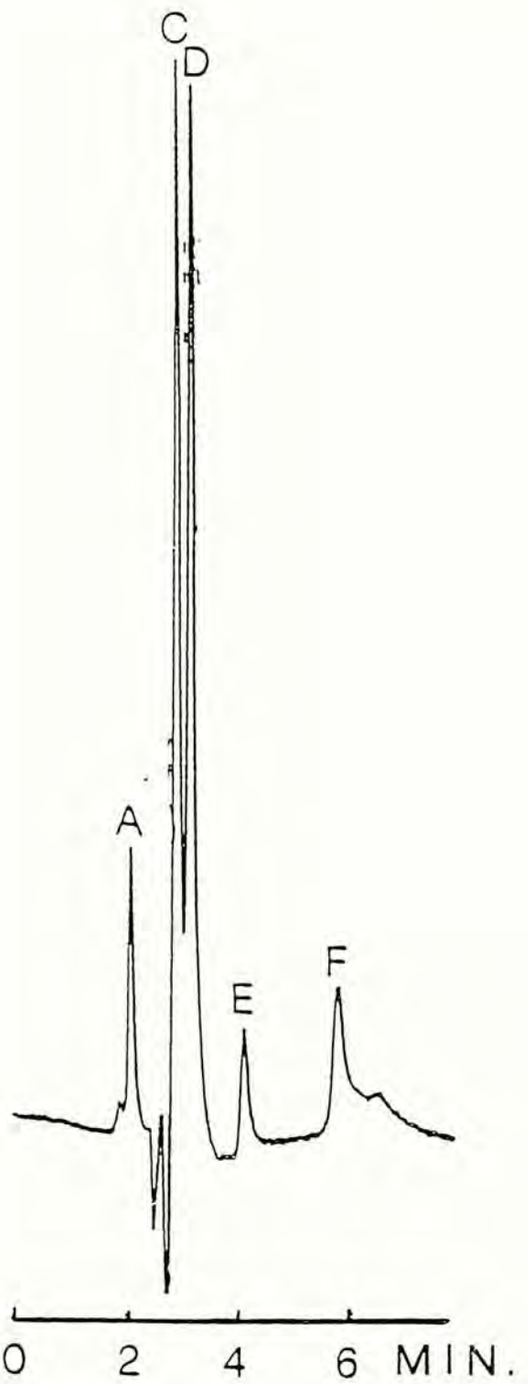


**Figure A.3.1-5** The HPLC chromatogram of a 333-fold diluted sample No.1 with peaks of excipients (A, D, E, and F) and peak of guaiphenesin (C).



A.3.2 The wavelength for detection

When the sample was diluted to 300-fold, the concentrations of guaiphenesin would be lowered to 15 - 25 ppm. However, the original wavelength for detection (i.e. 254 nm) was not at the absorbance maximum of guaiphenesin, and not sensitive enough to assay guaiphenesin at such low concentrations. Figure A.3.2-1 shows a UV spectrum of a standard guaiphenesin solution in the mobile phase, which indicated that the maximum absorbance wavelength of guaiphenesin in the mobile phase was at 274 nm. In order to enhance the signal of guaiphenesin, the wavelength of the UV detector was set at 274 nm. A typical chromatogram of standard guaiphenesin obtained at the 274 nm was shown in Figure A.3.2-2. The chromatograms of a real sample obtained using the original wavelength of 254nm and new wavelength of 274nm are shown in Figure A.3.2-3 and Figure A.3.2-4, respectively. The signal of guaiphenesin (Peak G) at the new wavelength was much higher. Besides, the resolution between guaiphenesin and other excipients was also improved using the new wavelength.

**Figure A.3.2-1** The UV spectrum of standard guaiphenesin (30.24 ppm) in the new mobile phase.

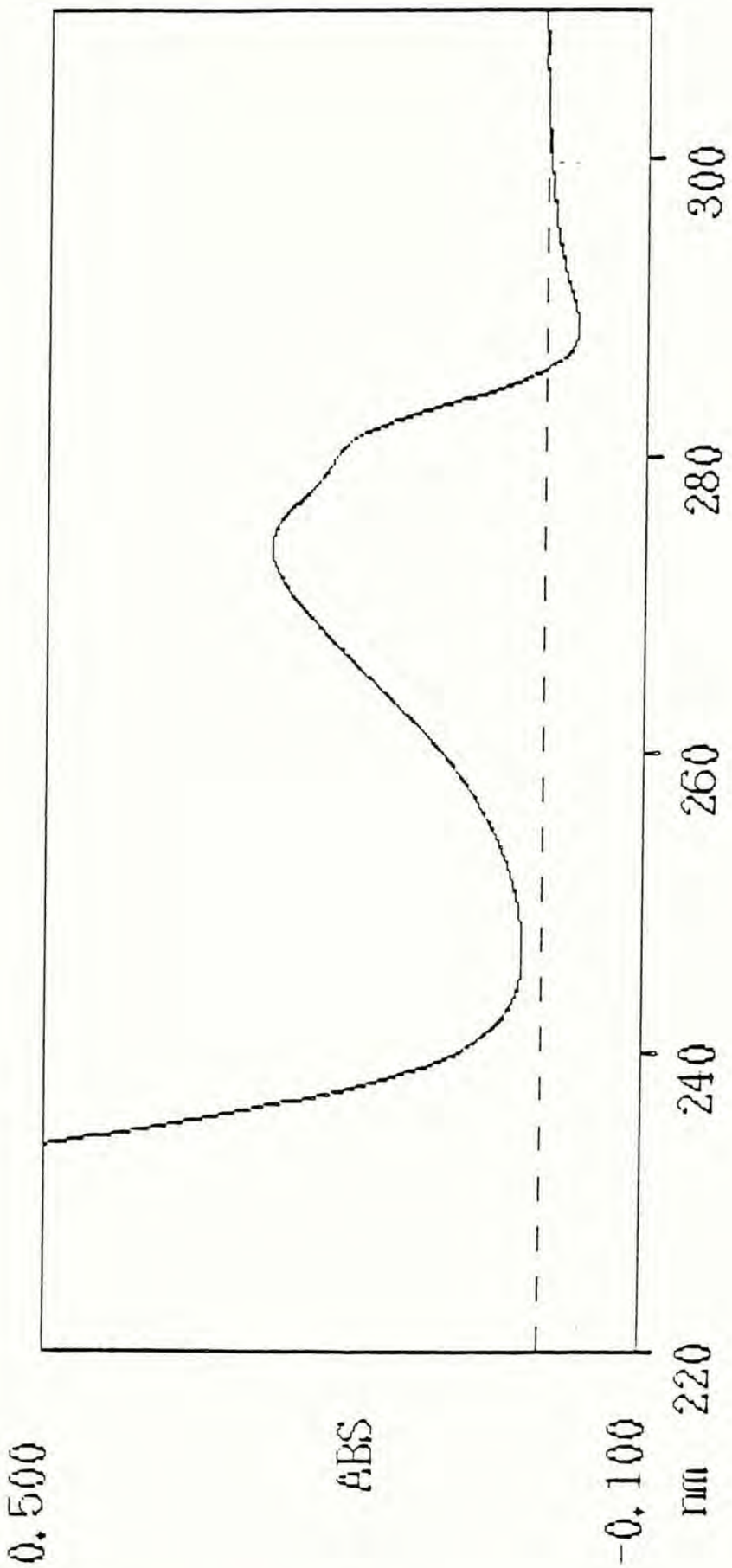


**Figure A.3.2-2** A typical HPLC chromatogram of standard guaiphenesin (25.20 ppm).

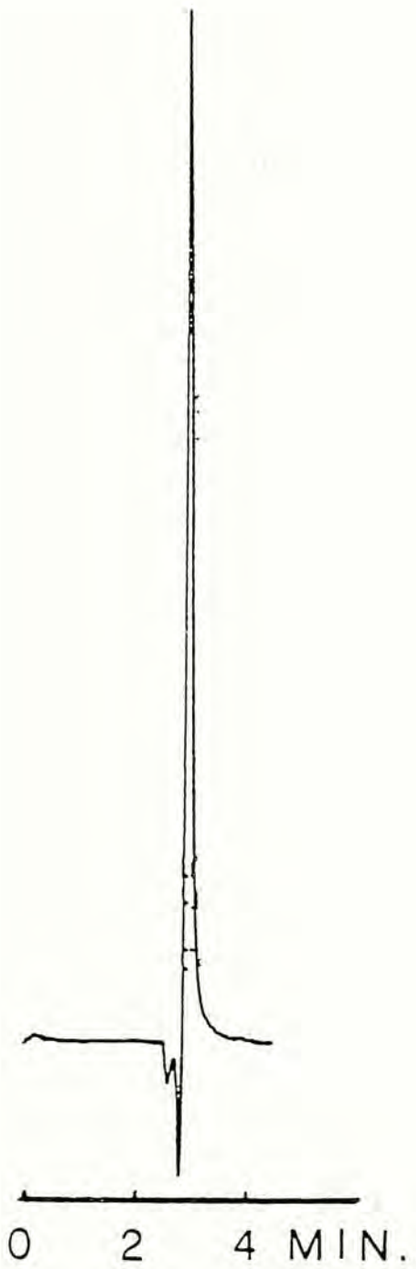
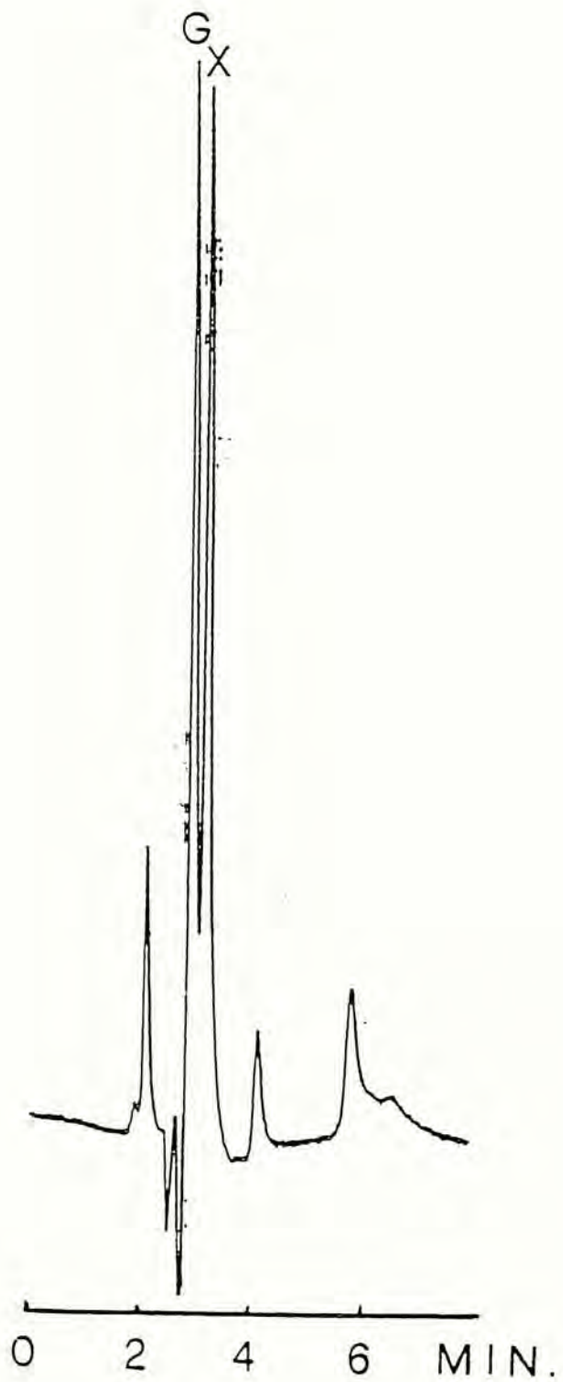


**Figure A.3.2-3** The HPLC chromatogram of 333-fold dilution of sample No. 1 obtained using the original wavelength of 254 nm, with peak of guaiphenesin (G).

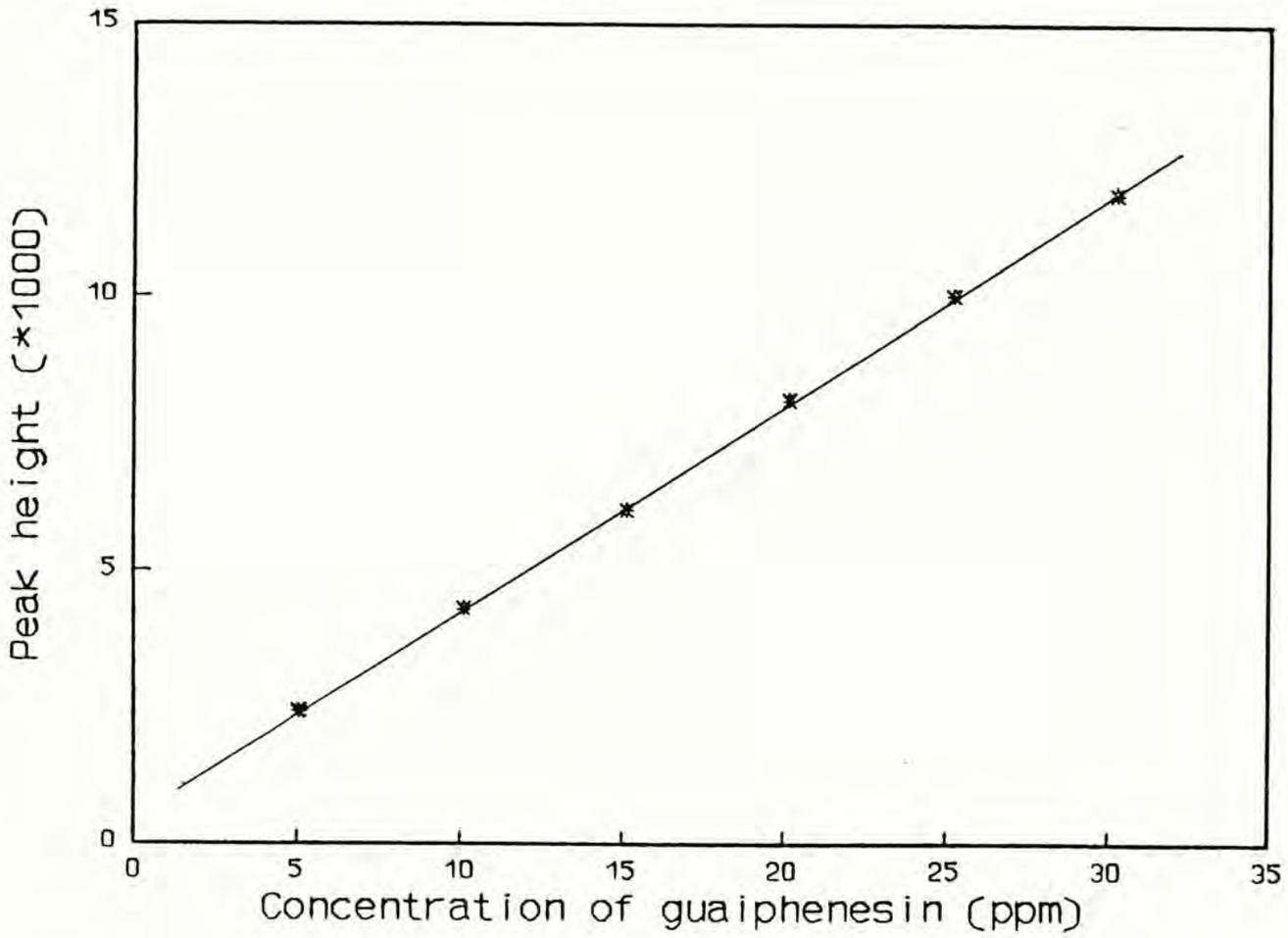
**Figure A.3.2-4** The HPLC chromatogram of 333-fold dilution of sample No. 1 obtained using the new wavelength of 274 nm, with peak of guaiphenesin (G).



A.3.3 Calibration graph

A calibration graph of guaiphenesin was plotted with the peak height of the compound against its concentrations using the proposed method, and the graph is shown in Figure A.3.3-1. With the help of the linear regression analysis, the slope of the graph was found to be 376.9 $ppm^{-1}$ and the correlation coefficient of the graph was 0.99992.

**Figure A.3.3-1** The calibration graph of guaiphenesin obtained using the modified method.



A.3.4 Real sample analysis

Five cough-cold syrups containing guaiphenesin, which had been previously analysed by the proposed GC method, were quantitatively determined using the modified method. The results are presented in Table A.3.4. A typical chromatogram of sample No. 1 is shown in Figure A.3.2-4. It was found that the peak of guaiphenesin was well separated from peaks of other excipients, except peak X. In order to minimize the interference due to peak X in measuring the signal of guaiphenesin, the peak height of guaiphenesin was measured instead of its peak area. The assay results agreed with those obtained by the GC method described in Chapter 2 and also the label claimed values.

**Table A.3.4-1** The assay results for the determination of guaiphenesin in cough syrups.

| Sample no.* | Labelled value (mg/5ml) | Percentage of label claim | | |
|---|---|---|---|---|
| | | By modified HPLC method | | By proposed GC method** |
| | | Amount found | Mean** | |
| 1 (2) | 25.0 | 91.7<br>93.7<br>94.5 | 93.3 (1.2) | 96.5 (1.5) |
| 2 (3) | 25.0 | 96.2<br>98.0<br>99.3 | 97.8 (1.3) | 98.5 (1.3) |
| 3 (7) | 100.0 | 102.5<br>99.1<br>100.3 | 100.6 (1.4) | 103.1 (1.3) |
| 4 (8) | 100.0 | 115.3<br>113.1<br>113.3 | 113.9 (1.0) | 111.4 (1.7) |
| 5 (9) | 100.0 | 99.4<br>102.3<br>102.9 | 101.5 (1.5) | 97.3 (0.5) |

*The sample number used in previous GC analysis are enclosed in parentheses.

**Mean of triplicate measurements, and standard deviation enclosed in parentheses.

## CONCLUSION

An established HPLC method, which was originally not suitable for the quantitative determination of guaiphenesin in cough-cold syrups, was found to be applicable in analysis of guaiphenesin in cough-cold syrups after minor modifications of the detector wavelength, dilution factor of the sample solution and the content of the mobile phase. High dilution factor (300-1000 folds) of the sample solution could minimize the interference due to the dyes. The signal of guaiphenesin was maximised by changing the wavelength from 254 to 274 nm. In addition, the increased water content of the mobile phase also helped to improve the separation of the peak of guaiphenesin from those of other excipients.

The modified method offered the advantages of convenience and economy to the analysts because there is no need to change the column and the reagents, although some minor changes in the original method were necessary to suit the analysis of guaiphenesin.

## REFERENCE

1. Lau, O.W., Chan, K., Lau, Y.K., and Wong, W.C., *Journal of Pharmaceutical & Biomedical Analysis*, 1989, 7, 725.

## LEGENDS FOR FIGURES

cont'd **Figures**

## LEGENDS FOR TABLES

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